t-AML-like *MLL* Rearrangements are Induced by Etoposide in Primary Human CD34+ Cells and Remain Stable after Clonal Expansion

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Abbreviations: Topo II, topoisomerase II; MLL, mixed lineage leukemia; DSB, DNA double-strand break; NHEJ, non-homologous end-joining; chr.11, chromosome 11; chr.4, chromosome 4; FISH, fluorescence *in situ* hybridization; PTD, partial tandem duplication; IPCR, inverse polymerase chain reaction

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Abstract

Rearrangements involving the MLL gene on chromosome band 11q23 are a hallmark of therapy-related acute myeloid leukemias following treatment with topoisomeraseII poisons including etoposide. Therapy-related and de novo genomic translocation breakpoints cluster within a well-characterized 8.3kb fragment of MLL. Repair of etoposide-stabilized DNA topoisomeraseII covalent complexes may initiate MLL rearrangements observed in patients. We used a culture system of primary human hematopoietic CD34+ cells and inverse PCR to characterize the spectrum of stable genomic rearrangements promoted by etoposide exposure originating within an MLL translocation hotspot in therapy-related leukemia. Alterations to the region were observed at a readily detectable frequency in etoposide-treated cells. Illegitimate repair events after minimal repair included MLL tandem duplications and translocations, with minor populations of deletions or insertions. In stably repaired cells that proliferated for 10-14 days, the significant majority of illegitimate events were MLL tandem duplications, and several deletions, inversions, insertions, and translocations. Thus, etoposide promotes specific rearrangements of MLL consistent with the full spectrum of oncogenic events identified in leukemic samples. Although etoposide-initiated rearrangements are frequent, only a small subset of translocations occurs in cells that proliferate significantly.
Introduction

Genome integrity is controlled by multiple damage surveillance pathways, cell-cycle checkpoints, and repair mechanisms. Despite these safeguards, illegitimate repair of chromosomal double-strand breaks (DSBs), such as those produced by chemotherapy agents that target topoisomerase II (topo II), can promote chromosomal rearrangements and, ultimately, tumorigenesis. Topo II is an essential cellular enzyme that catalyzes changes in DNA topology via its cleavage-religation equilibrium. Topo II targeted drugs that are poisons of this enzyme stabilize topo II-DNA covalent complexes, most often by decreasing the rate of religation in a dose-dependent manner. Disruption of the cleavage-religation reaction results in accumulation of DSBs, p53 activation, and induction of apoptosis or repair. Chromosomal DSBs are potent inducers of recombination stimulating the exchange of homologous sequences between two DNA duplexes 1000-fold not only between sister chromatids, but also between homologs, and sequence repeats on heterologous chromosomes. DSBs may be repaired by homologous recombination or non-homologous end joining (NHEJ), and both repair mechanisms have been associated with chromosomal translocations, a hallmark of leukemias, lymphomas and soft-tissue sarcomas.

There is clear evidence that exposure to chemotherapy or irradiation can result in subsequent development of therapy-related leukemias, which occur in 1–15% of patients exposed to DNA damaging agents in anti-cancer regimens. Exposure to topo II poisons such as etoposide is predominantly associated with therapy-related leukemias characterized by rearrangements of the MLL gene on chromosome band 11q23. MLL spans 100 kb, encodes a 430 kD protein homologous to the Drosophila trithorax gene, and has important functions in embryogenesis and hematopoiesis. MLL is a critical transcriptional regulator and numerous translocations involving MLL suggest that gain of function contributes to the critical leukemogenic lesion.

The MLL gene is involved in rearrangements and translocations with multiple partner genes, many of which are uncharacterized. Most MLL aberrations initiate within a particular 8.3 kb BamH1 fragment of the gene located between exons 5 and 11 known as the breakpoint cluster region (bcr). The European Union’s Concerted Action Workshop examined the spectrum of MLL rearrangements in patient samples. 16% were internal duplications, deletions and inversions, while the remaining 84% were reciprocal translocations with as many as 40 different partner genes. This particularly broad spectrum of rearrangements is a hallmark of MLL rearrangements and may reflect an inherent recombinogenic nature of the locus, or the central role of the MLL protein in proper hematopoietic development and differentiation such that rearrangements often possess...
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leukemogenic potential. Individual genetic factors have also been identified to predispose some patients to therapy-related leukemia with MLL alterations. However, the mechanisms that lead to specific rearrangements remain unclear. Etoposide-associated genomic alterations may coincide with topo II cleavage activity particularly within MLL. Etoposide addition to in vitro topo II cleavage assays enhances DNA topo II cleavage complexes within MLL and partner genes near translocation breakpoint sites identified in treatment-related leukemias. Topo II cleavage complexes with the MLL bcr in hematopoietic progenitor cells are detectable after etoposide exposure. Rearrangement of this region can be detected by Southern blotting after 16-24 h of continuous etoposide exposure in many hematopoietic cell lines. MLL bcr cleavage and rearrangements also are detectable following exposure of cells to multiple non-genotoxic agents, possibly due to apoptotic DNA fragmentation.

The observation that leukemias with MLL rearrangements are of both myeloid and lymphoid lineages and analysis of their gene expression profiles suggest that the MLL rearrangement and disease itself initiate within an undifferentiated hematopoietic stem cell. However, there are minimal data on the clastogenic effects of etoposide on the hematopoietic CD34+ stem cell-enriched population, the emergence of stable MLL rearrangements, or the proliferative potential of cells that contain illegitimate repaired rearrangement products. To provide direct evidence for stimulation of MLL rearrangements by etoposide in primary human CD34+ cells, we established an in vitro cell culture system of etoposide exposure and recovery, and used inverse PCR (IPCR) to directly determine the potential of etoposide exposure to lead to stable genome rearrangements originating within an MLL translocation breakpoint hotspot in therapy-related leukemia. Alterations to this region in etoposide-treated cells were readily detected. The spectrum of illegitimate repair products detectable after minimal 2-3 hr recovery included frequent MLL partial tandem duplications (44%; PTDs) and translocations (44%), with minor populations that contained deletions or insertions. However, in stably repaired cells capable of continued proliferation for 10-14 days, most illegitimate repair events were MLL PTDs (79%), and also included deletions, inversions, insertions, and translocations. Several clones had breakpoints that localize to MLL bcr sequences identified at rearrangement junctions in therapy-related leukemias. These data indicate that although etoposide-initiated rearrangements of MLL are frequent, only a small subset of chromosomal translocations occurs in cells that are capable of continued proliferation and, ultimately, leukemogenesis.
Material and Methods

Cell culture CD34+ cells were isolated from human umbilical cord blood specimens on ficoll gradient and positive selection through anti-CD34+ columns (Miltenyi). The purity of isolated CD34+ cells was determined by phycoerythrin (PE)-conjugated anti-CD34+ antibody (anti-HPCA-2, Becton Dickinson) and flow cytometry. Alternatively, frozen adult peripheral blood CD34+ cells were obtained from NCI core facility, Seattle, WA. Other cell lines obtained from ATCC included TF-1 (RPMI supplemented with glutamine (GIBCO), 10% FBS), WS1 fibroblasts (DMEM, 10% FBS), M059K, and M059J (DMEM/HAM-F12, 10% FBS). Informed consent was provided according to the Declaration of Helsinki. Use of human cells was approved by Columbia University Institutional Review Board (IRB # 15183). Cells were cultured in Iscove’s IMDM media supplemented with 25% BIT9500 (Stem Cells Technology), Flt-3L, TPO, 100 ng/mL SCF (Peprotech) for 2 – 4 days. CD34+ cells were exposed to 20 – 50 µM etoposide (Sigma-Aldrich; 20 mM stock solution prepared in dimethylosulphoxide (DMSO)) 1 hr and recovered in myeloid differentiating media (IMDM, BIT9500, 10 ng/mL IL-3, 10 ng/mL G-CSF, 10 ng/mL GM-CSF (Peprotech)). Myeloid differentiation was confirmed after 7-14 days by PE-conjugated anti-CD11b antibody (Becton Dickinson) or anti-PE-conjugated anti-CD34+ antibody.

Analysis of DNA damage, apoptosis and recovery PFGE was performed to detect high molecular weight DNA fragmentation. 200,000 cells were immobilized in 1.5% low melting point agarose. Plugs were treated with extraction buffer (10 mM Tris-HCl, pH 9.5; 10 mM NaCl, 25 mM EDTA, 1 mM ethylene glycol – tetraacetic acid (EGTA), 1.5% SDS, 0.1% β-mercaptoethanol), washed three times in 10 mM EDTA, 1 mM Tris-HCl (pH 8) and high molecular weight DNA was resolved on a 1% 0.5X TBE agarose gel using the FIGE mode of the CHEF Mapper™ (BioRad). DNA entry into the gel was facilitated by continuous electrophoresis for 20 minutes at 6 V/cm. Field inversion followed at 5 V/cm, a 3:1 ratio of forward to reverse switch times, linearly ramping 16 hr at 14 °C. Fragmentation was viewed after ethidium bromide staining. Apoptosis was evaluated using an annexin V-FITC apoptosis detection kit (Pharmingen). Cell survival and proliferative recovery was assessed using Alamar Blue (Biosource) or counting by hemacytometer.

IPCR Genomic DNA was digested with Xba I, extracted with phenol-chloroform, and ethanol-precipitated. 5 ug DNA fragments were circularized with 2000 units T4 DNA ligase in 300 µL at room temperature. DNA was purified by phenol-chloroform extraction using phase lock-gels (Eppendorf) followed by ethanol-precipitation. 200 ng DNA was used for the first PCR. 1 µl of the first PCR was used for nested PCR. Final PCR products were separated on a 1% agarose gel. Two sets of nested primers were used to amplify MLL and putative fusion partners: MF1-5’TCTACAAGTGCCAGGGGTCT3’; MF2-
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5’AATAGCATGCTGCTGCCTGCACCTGACTCTCTAA3’, MR1-
5’CCCGAGTGGATTGTTTTTTTA3’; MR2-
5’GATCGTAGGATATGTCCCTTATAAATGACAAACTACTGCTTCC3’. First PCR: 35 cycles of 94°C – 30 sec/54°C – 45 sec/68°C – 3 min + 20 sec each cycle after the 10th cycle. Second PCR: 35 cycles of 94°C – 30 sec/56°C – 45 sec/68°C – 3 min + 20 sec each cycle after the 10th cycle. In some cases, Pvu II digestion of DNA prior to PCR eliminated amplification of competing germline product. The entire PCR reaction, or individual IPCR products excised and purified from gels, was cloned into pCR2.1 TOPO (Invitrogen). Resultant cloned PCR products were sequenced with M13 forward and reverse primers, and, for larger products, with internal MLL-specific primers. Nucleotide sequences were compared with MLL and analyzed using NCBI BLAST.

Chromosome analysis
Cells were incubated with ethidium bromide (10 µg/mL) 15 min then Colcemid (GIBCO) 2 hr. Cells were swelled in hypotonic 0.54% KCl solution, and fixed in methanol-acetic acid (3:1) three times. Metaphase spreads were analyzed by fluorescence in situ hybridization (FISH) using chr. 11 and chr. 4 whole chromosome probes according to manufacturer’s protocol (Vysis). Aberrations were scored in approximately 100 metaphases. In parallel, metaphase and interphase spreads were analyzed by FISH using an MLL locus-specific identifier split-probe (LSI; Vysis) according to manufacturer’s protocol. Approximately 250 metaphases or interphases were scored per sample.

Results

Human CD34+ cell etoposide exposure
We established an in vitro culture and IPCR system to directly determine the potential of etoposide exposure to lead to stable genome rearrangements originating within the MLL locus on chromosome band 11q23 in primary stem-cell enriched CD34+ cells. Fresh umbilical CB CD34+ cells were isolated by ficoll gradient and positive selection through anti-CD34+ columns. Peripheral blood CD34+ cells from adult individuals were obtained from NCI. Staining of cells immediately following isolation with PE-conjugated anti-CD34+ antibody and analysis by flow cytometry confirmed >90% purity in all samples (data not shown). Although CB cells represent an immature population and possibly contain cells with higher replication and repair potential, both cell sources represent similar mobilized peripheral blood CD34+ cells induced by stress conditions and cytokines, and both gave similar results as discussed below. Depending on yield, between 5 and 8 individual samples were pooled to obtain 3 – 5 x 10^6 cells per experiment, and 4 independent experiments were performed. CD34+ cells were cultured for 2-4 days in IMDM supplemented with SCF, TPO, and Flt-3L to optimally maintain and expand stem cells and progenitors. To determine
the clastogenic effects of etoposide, CD34+ cells were exposed for one hour with a physiologically relevant dose of 20-50 µM etoposide, in agreement with pharmacokinetic studies demonstrating peak plasma levels in patients of 25-75 µM. Etoposide was subsequently washed away, and cells were allowed to repair DNA damage and proliferate. Following 7-14 days expansion in culture, flow cytometry confirmed that the majority of cells were CD34- and, on average, 40% were CD11b+, indicating substantial myeloid differentiation during this period (data not shown). We did not examine directly the percentage of lymphoid cells in expanded populations. However, culture conditions used were expected to greatly favor myeloid differentiation.

The overall level of DNA damage, chromatin fragmentation, and cell viability following etoposide was assessed by pulse field gel electrophoresis (Fig. 1A), and annexin V staining (Fig. 1B). >400 kb fragments were immediately visible after 1 hr of etoposide exposure. This population disappeared within 2-4 hr after removal of etoposide, either due to repair of DNA damage or due to apoptosis and clearance of dead cells from the population. By 8 hr after etoposide removal, 50 kb fragments were detected, corresponding to DNA fragmentation by caspase-activated deoxynucleases and early apoptosis in a portion of treated cells. 20-60% of cells were viable 24-48 hr after treatment (Fig. 1B), depending on the individual sample (data not shown). Incubation of cells for 10-14 days demonstrated recovery of proliferative capacity of the remaining viable cells. By 14 days, numbers of control and etoposide-treated cells increased 155-fold and 150-fold, respectively (Fig. 1C). As expected, continuous exposure of CD34+ cells to 20 µM etoposide for 24 hr resulted in decreased cell survival (0 – 5%) and significantly more prominent >400 kb and 50 kb band formation as well as decreased cell viability (Fig. 1A). Although there is likely an etoposide dose response effect in CD34+ cells, these cells were not further analyzed.

**IPCR analysis of etoposide-induced MLL repair products**

To determine the direct potential for etoposide to lead to MLL rearrangements and the stability of rearrangements in viable cells, cells were harvested either 2-3 hr (short recovery period) or 10-14 days (long recovery period) after removal of etoposide. We used IPCR to determine the fidelity of repair in a 1.8 kb region in the 3' portion of MLL intron 8 that contains a translocation breakpoint hotspot in treatment-related leukemia and exon 9 that contains sequence homology to a putative topo II recognition sequence and is sensitive to DNaseI and multiple cytotoxic agents (Fig. 2A). IPCR of untreated samples gave almost exclusively a germline 1.8 kb product that was confirmed by sequencing (Fig. 2B). In contrast, IPCR of etoposide-treated cells also gave readily detectable variable-sized bands representing alterations to the region (Fig. 2B). As visualized by gel electrophoresis (Fig. 2B), IPCR favors amplification of smaller rearranged products (300 -700 bp) with some larger (1.5 - 2 kb) products; thus, the total number of rearrangements in each etoposide-
Etoposide-induced stable *MLL* rearrangements treated population is likely higher. More clones were observed from IPCR reactions harvested after a short recovery as compared to samples harvested after a long recovery. Any decrease in detectable illegitimate repair products between short and long recovery times is consistent with chromatin fragmentation and apoptosis of cells with unrepaird damage 12-24 hr after etoposide exposure, but could also reflect competitive expansion of specific clones over time. Parallel experiments in multiple hematological and other adherent cell lines gave similar IPCR results (Fig. 2B), but individual clones were not characterized.

We isolated, cloned and sequenced 67 novel IPCR products from primary CD34+ cells to characterize etoposide-induced illegitimate repair events. 34 were from samples harvested after the short recovery period, and 33 were from samples harvested after the long recovery period (Table 1). After short recovery, half of the analyzed repair events (16 of 34, 47%) were intragenic *MLL* rearrangements. Almost all of these events were PTDs. The most extensive PTD contained a fusion between intron 8 and intron 4, 5′ of the *MLL* bcr. One product contained a deletion of more than 600 bp. The other half of repair products (18 of 34, 53%) contained fusions of *MLL* to new sequence. The majority of these (15 of 18) were apparent chromosomal translocations. These events are in agreement with other reports that DNA damaging agents induce *MLL* translocations immediately after exposure and a short recovery period. The remaining 3 events contained insertions of novel sequences ranging from 39 bp to 180 bp.

By contrast, after a long recovery almost all analyzed repair events (31 of 33, 94%) were intragenic *MLL* rearrangements. Similar to products analyzed after short recovery, most of these were PTDs (26 of 33, 79%). In addition, one product contained a deletion of approximately 100 bp, and 4 were inversions of sequence within the *MLL* bcr. Only a small minority of products (2 of 33, 6%) contained fusions of *MLL* to new sequence; one contained an insertion of 180 bp, and one was an apparent translocation. Thus, products derived from etoposide-treated cells and repaired within 2-3 hr showed a significantly higher proportion of translocations in comparison to the spectrum of repair products isolated from cells that proliferated for 14 days (53% versus 6%, p=0.00002). As an additional control for PCR contamination or artifact within samples, multiple aliquots of isolated DNA from the same sample were used for independent IPCR amplifications. In several cases we cloned and sequenced the same repair products from two different aliquots of the same sample, and in one case, the same repair product was present in three different aliquots of the DNA sample. Identification of these events in multiple aliquots after a long recovery is further evidence that they are contained within viable and proliferating cells.

Overall, the breakpoint junctions were similar to breakpoints sequenced from leukemic cells. Several cloned rearrangement junctions localized to *MLL* bcr sequences that have been
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were present at approximately 70% of the breakpoint junctions (Fig. 3). In 3 cases a longer patch of overlapping homologous sequence of 21 bp, 277 bp, and 350 bp was observed at the breakpoint junction, suggesting single-strand annealing as the DNA repair mechanism. The distribution of etoposide-induced breakpoints in the repair products is displayed against the localization of repetitive elements within the MLL bcr in Fig. 4. 86% of PTD breakpoints and 60% of translocation breakpoints localized within repetitive elements. 31 of the 35 PTD breakpoints contained Alu-Alu or LINE-LINE fusions, supporting previous reports of repetitive element-mediated recombination to generate MLL PTDs in AML. Sixteen of the 20 novel sequences fused to MLL were of sufficient length to identify by BLAST. Apparent translocations fused MLL to sequence located on chromosome bands 3q26, 3p25, 4q31-32, 4p15, 6q21, 7q31-32, 9q21-22, 12q21 (2 clones), 14q21, 15q12-13, 17q24 (3 clones), and Xq22-23. Although several of these chromosomal bands contain known partner genes of MLL (3q25-26 – GMPS, 6q21 - AF6q21, 17q24 - LASP1, Xq22-23 - Septin6), the partner sequences in the induced clones did not match known MLL partners.

Karyotypic analysis of etoposide-induced chr. 11 aberrations

We visualized aberrations of the MLL locus at the chromosomal level by FISH using a dual-color fluorescent split signal MLL probe (Fig. 5A). This technique captures all structural aberrations of the MLL bcr corresponding to fragmented chromosome material including translocations, dicentrics, amplification, and loss of MLL sequence. Three independent primary CD34+ samples were divided into parallel untreated or etoposide-treated aliquots, cultured and proliferated for 7-14 days, and harvested on the same day. At least 190 metaphase spreads from each sample were scored. In untreated samples, 4 of 640 cells separated the overlapping fluorescent probes, and no cells contained multiple signal aberrations (0 of 640). By contrast, following etoposide exposure and proliferation for 7-14 days, 59 of 850 cells separated the overlapping fluorescent probes, indicating that they contained MLL translocations (p-value<10⁻¹¹; Fig. 5, compare F with G, H). In some cases the second fluorescent signal was not visible. These events could be either translocations involving loss of a significant amount of chromosome band 11q23 material during the translocation event or deletions of the whole chr. 11 end. Interestingly, 46 of 850 cells contained multiple signal aberrations (p-value<10⁻¹³; Fig. 5, compare F with I, compare J with K-M) indicating that etoposide treatment can promote complex MLL locus rearrangements within a single cell, which is consistent with complex MLL rearrangements that occasionally occur in patients. The percentage of gross chromosomal translocations that included chr. 11 was confirmed by FISH using a chr. 11- Cy3 probe (Fig. 5, B-E). Because a predominant MLL translocation fusion partner is AF-4 located on chromosome band 4q21, we compared the frequency
Etoposide-induced stable MLL rearrangements of translocations that included chr. 4 using a chr. 4-FITC probe (Fig. 5, B-E). Following etoposide exposure and long recovery, translocations involving chr. 11 were observed in 10% of metaphases, and translocations involving chr. 4 were observed in 5% of metaphases. In no case, did we observe a t(4;11). However, the cell culture conditions favored myeloid expansion and the t(4;11)(q21;q23) fusing MLL and AF-4 occurs mainly in ALL and less often in monoblastic AML. Aberrations involving either chromosome were observed in 1% of untreated controls, in agreement with the expected frequency of spontaneously detectable aberrations of cells in culture.\(^{63,64}\)

**Discussion**

We established an *in vitro* culture system and IPCR to characterize the spectrum of stable, and potentially leukemogenic, genomic rearrangements initiating within a therapy-related translocation hotspot of the MLL bcr induced by etoposide exposure. IPCR of etoposide-treated CD34+ cell samples gave readily detectable, variable-sized bands representing alterations to the region. Importantly, in several instances, the same aberrations were obtained in different aliquots of a single DNA sample used for independent amplifications. In samples that repaired DNA damage and proliferated 10-14 days, this result is likely due to expansion of aberrant clones. However, in samples harvested 2-3 hr after etoposide exposure when no proliferation of cells is expected, this result suggests that some sequences are preferential substrates for etoposide-induced cleavage and illegitimate recombination repair events. We estimate that the relative frequency of the stable MLL illegitimate repair products is 2-4 per 20,000 etoposide-treated cells (1.5 x 10\(^{-4}\)). This calculation is based on the relative sensitivity of PCR to amplify 1 copy per 10,000 cells, and 200 ng DNA used per PCR reaction corresponding to 40,000 cells, and 50% cell death induced by etoposide at this dose range.

Our results imply that the initial formation of MLL locus aberrations including translocations are a general phenomenon, and can develop independently of individual genetic factors; however, any dysfunction or variation of DNA damage sensor and repair proteins might be expected to influence both the frequency and spectrum of repair products. After etoposide removal, DSBs rapidly disappear in 2-4 hr, and, within this short period, we detected multiple rearrangements with a high ratio of MLL translocations. The major decrease in cell number occurred 12-24 hr after treatment, presumably as cells with unrepaired DNA breaks led to apoptosis and cell death. Cells that appropriately repaired damage or acquired non-lethal DNA rearrangements resumed proliferation after 2-4 days in culture. However, the development and final emergence of a malignant clone responsible for therapy-related leukemia depends on the survival and proliferative potential and possibly secondary alterations leading to the selection and clonal...
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expansion of the cell in which repair has occurred. The early clearance and delayed selection of
rearranged clones is consistent with the development of \textit{MLL}-rearranged therapy-related leukemias
in a small fraction of patients receiving chemotherapy regimens that include topo II poisons.

The specific etoposide-induced rearrangements initiating within \textit{MLL} reported here are
consistent with the full spectrum of tandem duplications, translocations, and complex
rearrangements identified in cells from patients with therapy-related leukemias\textsuperscript{55,65,66}. The
spectrum of illegitimate repair products detectable after minimal 2-3 hr recovery included frequent
\textit{MLL} PTDs and translocations. However, in cells that repaired damage and continued to proliferate
for 10-14 days, the significant majority of illegitimate repair events were \textit{MLL} PTDs, and a single
translocation. This shift in the spectrum of detectable rearrangements may result from clearance of
unstable clones through apoptosis or competitive expansion of certain clones due to proliferative
advantage. Although our data indicate that stable \textit{MLL} PTDs are a frequent outcome of etoposide-
induced DNA damage, it is likely that many are not fully leukemogenic, and that additional
alterations are needed. Smaller duplications would not be expected to alter MLL protein function,
but produced mechanistically similar to those in leukemias in patients\textsuperscript{54}. Since our culture
conditions favor expansion of myeloid precursors, PTDs also seem consistent with the clinical
association of \textit{MLL} PTDs with AML but not ALL. Conditions generating lymphoid precursors
might result in expansion of clones with different spectrum of products.

The low ratio of deletions is likely due to the predominant isolation and analysis of
products that differed significantly in size relative to the genomic 1.8 kb \textit{MLL} fragment. The
smallest deletion was 100 bp, similar to another study in which small deletions of 100-200 bp of the
\textit{TEL} gene locus were detected in 12 of 17 clones from ALL cell lines after serum starvation\textsuperscript{67}.
Larger deletions that include the primers used for IPCR would also be excluded from this analysis.
Alternatively, the lack of deletions could reflect locus and chromosome specific differences or
differences in the preferred type of DNA damage induced by etoposide. This study also revealed 3
complex repair events with insertion of foreign sequences followed by a PTD of \textit{MLL} sequence. A
similar sequence abnormality was described in a case of B-lineage ALL in which a duplicated
portion of \textit{MLL} was interrupted by a 72 bp insertion of \textit{AF9} genomic sequence from 9p22\textsuperscript{68}.
Together with the complex events detected by cytogenetic analysis, these data indicate that the
spectrum of potentially leukemogenic \textit{MLL} repair events detected in this experimental system are
similar to events identified in leukemia in patients. Additional studies in patients undergoing
treatment would determine whether there is also a shift in repair products from early times
following etoposide exposure to when stable leukemia-associated clones have been established.
FISH analysis correlated with the molecular analysis. FISH analysis using the *MLL*-specific probe identified aberrations within this locus in 12% of cells (105 of 850). Similarly, using FISH and whole chromosome fluorescent probes, we detected chr. 11 aberrations twice as frequently as chr. 4 aberrations. This is in accordance with results of cytogenetic analysis of peripheral blood mononuclear cells after 48 hr continuous exposure to etoposide in which chr. 11 was altered two times more frequently than expected based on relative length, and chr. 4 was altered at a lower frequency than expected. This result also is consistent with the predilection for translocations involving chr. 4 to occur in cells of lymphoid lineage and the culture conditions used here that would favor myeloid outgrowth.

Among the observed intragenic rearrangements most were PTDs with breakpoints localized within repetitive elements, which were detected more frequently in this model system than seen in patient samples. Alterations of the *MLL* locus in leukemic cells have been found to involve PTDs at or near Alu repeats. It has been proposed that Alu elements promote homology-directed replication slippage or homology-mediated illegitimate DSB repair between sister chromatids or homologs. A similar repair mechanism could account for both leukemia-associated *MLL* PTDs and those induced in this experimental system. PTDs have not been detected in other reports of human cells treated with etoposide or after apoptotic stimuli, although others have not analyzed cells after extensive proliferation or events at the sequence level. A recent report on rearrangements of the *MLL* locus in mouse embryonic stem (ES) cells does not provide evidence of etoposide-induced PTDs in *MLL* after 48 hr recovery. This difference might be due the sequence divergences between the *MLL* locus of the two species, the general lack of Alu elements in the rodent genome, or different repair pathways employed for DSB repair in ES cells compared to hematopoietic CD34+ progenitors. Homologous recombination is a predominant DSB repair pathway in ES cells, but the relative reliance of repair pathways in the stem cell-enriched CD34+ cell population is not known. However, mouse ES cells can repair I-SceI endonuclease-induced DSBs to form stable intragenic duplications and chromosomal translocations between introduced neomycin gene sequence repeats (frequency 1 x 10^-4), indicating that ES cells and hematopoietic progenitors can use the same repair pathways in at least some instances.

It is notable that a significant majority of both the PTD breakpoints (86%) and translocation breakpoints (60%) localized within repetitive elements (Fig. 4). The *MLL* bcr contains 8 Alu, 4 LINE, and 2 low copy MER repetitive elements that span 3824 bp (46% of the bcr). Thus, the percentage of both internal rearrangements (86% vs 46%) and translocations (60% vs 46%) located within repetitive elements is higher than expected by chance. The presence of Alu elements previously identified at or near breakpoint junctions in leukemic cells suggested that such
sequences are capable of illegitimate invasion into a homologous Alu sequence during DSB repair or restart of a collapsed replication fork. 31 of the 35 recovered intragenic duplications contain Alu-Alu or LINE-LINE element breakpoint junctions, demonstrating experimentally that homology-mediated invasion is a predominant mechanism to repair etoposide-stimulated DNA damage. Further, the spectrum of repair products identified immediately after etoposide exposure demonstrates that illegitimate homology-mediated repair can occur with similar frequency between homologs or sister chromatids as between heterologs. Repair mechanism choice may be locus specific. In support of this, the MLL bcr fragment may act in cis to promote spontaneous recombination 3-4 fold between SV40 molecules that can be elevated by etoposide treatment.

Homology-mediated DSB repair in mammalian cells requires members of the RAD52 epistasis group of proteins. Rad51 is central to most homologous recombination events; Rad51 forms nucleoprotein filaments on ssDNA, and mediates homologous pairing and strand exchange between DNA duplexes, although single-strand annealing can be promoted by Rad52 in the absence of Rad51. Whether the homology-mediated events observed here are Rad51-dependent, or are sufficiently promoted by the annealing activity of Rad52 is not clear. Aberrant over-expression of Rad51 is tolerated for cell viability, but leads to multiple cell defects including impaired cell cycle progression, promiscuous recombination, and aneuploidy. Elevated levels of Rad51 detected in multiple tumor cell types may contribute to genomic instability by stimulating recombination between short repetitive elements and homologous sequences. Expression of Rad51 is predictive of B-CLL patient response to nitrogen mustards, as well as small cell lung cancer cell sensitivity to VP-16. Thus, the ability to modulate damage sensor and repair proteins will impact the ability of the CD34+ sub-population to protect against illegitimate repair of etoposide-induced DNA damage and potentially oncogenic rearrangements. This system can be used to determine the relative importance of repair proteins in the maintenance of genome stability following exposure of CD34+ cells to chemotherapeutic regimens that include etoposide.

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References
3. Abraham RT. Cell cycle checkpoint signalling through the ATM and ATR kinases. Genes @ Dev. 2001;15:2177-2196.
Libura, et al.

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17. Pedersen-Bjergaard J, Rowley JD. The balanced and the unbalanced chromosome aberrations of acute myeloid leukemia may develop in different ways and may contribute differently to malignant transformation. Blood. 1994;83:2780-2786.


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36. Stanulla M, Wang J, Chervinsky DS, Thandla S, Aplan PD. DNA cleavage within the MLL breakpoint cluster region is a specific event which occurs as part of higher-order chromatin fragmentation during the initial stages of apoptosis. Mol Cell Biol. 1997;17:4070-4079.
Etoposide-induced stable MLL rearrangements


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63. Pantelias GE, Maillie HD. Direct analysis of radiation-induced chromosome fragments and rings in unstimulated human peripheral blood lymphocytes by means of the premature chromosome condensation technique. Mutat Res. 1985;149:67-72.


**Table 1.** Spectrum of etoposide-induced *MLL* locus repair products in primary CD34+ cells.

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<td><strong>Internal <em>MLL</em> rearrangements</strong></td>
<td></td>
<td></td>
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<tr>
<td>Tandem duplication&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>26</td>
</tr>
<tr>
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<td>1</td>
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<tr>
<td>Inversion</td>
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<td>4</td>
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<tr>
<td><strong>Fusion to novel sequence</strong></td>
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<td></td>
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<tr>
<td>Insertion</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Translocation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total clones analyzed</strong></td>
<td>34</td>
<td>33</td>
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</table>

<sup>a</sup> P-values calculated by the Fisher’s two-tailed exact test. The increase in relative frequency of tandem duplications (p-value <0.006) is paralleled by the significant decrease in translocations (p-value<0.00002).

<sup>b</sup> Three of these events were complex with insertions of novel sequence at the translocation junctions.
**Figure Legends**

**Figure 1.** Cytotoxicity of etoposide on primary human CD34+ cells. 

A. Pulse field electrophoresis of genomic DNA to detect DNA fragmentation following 20 μM etoposide exposure and 0 hr to 24 hr recovery times. >400kb and 50kb fragment populations indicated on left. 


C. Recovery and proliferation of CD34+ cells in culture after exposure to etoposide. Three independent experiments were performed. The difference in expansion between the untreated and treated samples is statistically significant on days 1, 2, and 7. Grey bars-untreated CD34+ cells. Black bars: CD34+ cells following 20 μM etoposide exposure.

**Figure 2.** IPCR analysis to detect etoposide-induced rearrangements initiating within the MLL bcr. 

A. Schematic representation of the MLL gene locus on chromosome band 11q23. The 8.3 kb MLL bcr, flanked by BamHI sites, includes exons 5-11 and intervening introns. XbaI sites are 2.6 kb apart within the MLL bcr. Genomic DNA was digested with XbaI and circularized. Nested PCR reactions were carried out with F1-R1 primer pair, followed by F2-R2 primer pair. BH-BamHI; X-XbaI; F1, F2, R1, R2-primers used for PCR (see materials and methods). 

B. Representative IPCR products. Lanes 1-7: untreated controls that give the expected 1.8 kb germline product. Lanes 8-18: etoposide-treated samples that all give alternative products representing possible rearrangements of MLL. Pre-treatment of samples shown in lanes 8-18 with PvuII eliminated some or all detectable germline product to facilitate isolation of individual alternative sized products. Lanes 8-12: multiple independent CD34+ cell samples (long recovery) with all germline product eliminated by PvuII in Lanes 8 and 9; Lane 13: TF-1 (short recovery); Lane 14: WS1 (short recovery); Lane 15-16: M059K (short recovery); Lane 17: M059J (short recovery); Lane 18: cord blood mononuclear cells (short recovery). Parallel experiments in multiple hematological and other adherent cell lines gave similar IPCR results (Fig. 2B), but individual repair clones have not been fully characterized.

**Figure 3.** Representative etoposide-induced breakpoint sequence junctions. Left panel: representative sequence junctions of translocations of MLL gene to heterologous chromosomes. Red indicates sequence 3’ of the breakpoint junction, and green indicates sequence 5’ of the breakpoint junction. Right panel: representative sequence junctions of MLL PTDs. Underlined red sequences indicate overlapping homology between fusion partners. Bold black sequences or numbers indicate insertions. Numbers in brackets correspond to nucleotide lengths of longer
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insertions or homologies. For instance, in the top clone, a 2.1 kb PTD was produced following an
Alu-Alu fusion with 23 bp overlapping homology.

**Figure 4.** Summary of etoposide-induced breakpoints within the MLL bcr. Localization of
repetitive elements in 8.3 kb MLL bcr, and distribution of breakpoints associated with translocations
and insertions (above) or intragenic duplications (below). Alu elements in same orientation (grey
arrows; 799-1109, 1119-1420, 1423-1716, 1927-2215, 3973-4268, 4765-5094, 6072-6372, 7163-
7427), LINE elements (black arrows; 3490-3589, 3691-3972, 4269-4612, 5610-5998), low copy
MER repeat (green arrows; 6959-7162, 7428-7558). * - sequence homology to a putative topo II
recognition sequence (according to 22).

**Figure 5.** Fluorescence *in situ* hybridization to visualize etoposide-induced chr.11 rearrangements
in cells after long recovery period. A-C. Whole chromosome painting with chr. 11-Cy3 (red) and
chr. 4-FITC (green). A. Schematic overlap between fluorescent probes of 11q23 locus specific
identifier (LSI). Intact 11q23 is seen as yellow signal (overlap of green and red). Aberration of
11q23 is seen as separation of probes into independent green and red signals. B. Untreated CD34+
control. C-E. Representative metaphase spreads that contain chr.11 aberrations. F-I. LSI of
chromosome band 11q23 in interphase and metaphase cells. F and J. Untreated CD34+control. G
and H. Representative split signals observed in 59 of 850 etoposide-treated and analyzed cells. I,
K-M. Representative complex rearrangements observed in 46 of 850 etoposide-treated and
analyzed cells.
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<table>
<thead>
<tr>
<th><strong>MLL translocations</strong></th>
<th><strong>MLL intragenic tandem duplications</strong></th>
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<td><strong>MLL BCR 6094 bp</strong></td>
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t-AML-like MLL rearrangements are induced by etoposide in primary human CD34+ cells and remain stable after clonal expansion

Jolanta Libura, Diana J Slater, Carolyn A Felix and Christine Richardson