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Etoposide-initiated *MLL* rearrangements detected at high frequency in human primitive hematopoietic stem cells with *in vitro* and *in vivo* long-term repopulating potential

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Abstract

Rearrangements initiating within the well-characterized break-point cluster region of the mixed lineage leukemia (*MLL*) gene on 11q23 are a hallmark of therapy-related leukemias following treatment with topoisomerase II poisons including etoposide. Hematopoietic stem cells (HSC) are believed to be the target cell for leukemia-initiating *MLL* rearrangement events. Although etoposide treatment is sufficient to induce readily detectable *MLL* rearrangements in primary human CD34+ cells, the majority of cells that gain translocations do not proliferate in culture possibly due to reduced proliferative capacity of most CD34+ cells during normal differentiation [Blood 2005;105:2124]. We characterized the impact of etoposide on primary human long-term repopulating HSC that represent only a minor portion of CD34+ cells. The proliferative capacity of HSC is dramatically increased following both a single and multiple exposures to etoposide as determined by their ability to engraft bone marrow of immune-deficient non-obese diabetic/severe combined immunodeficient mice and to initiate hematopoiesis in long-term initiating cultures. Similar to results in CD34+ cells, a significant proportion of etoposide-treated HSC-derived clones harbored stable *MLL* rearrangements, including duplications, inversions and translocations. These results indicate HSC are highly susceptible to etoposide-induced and potentially oncogenic rearrangements initiating within *MLL*, and these HSC are particularly proficient for continued long-term proliferation both *in vivo* and *in vitro*.

Keywords

etoposide; rearrangements; primitive hematopoietic stem cells; CD34+ cells; Alu repetitive elements; genome instability; therapy-related leukemia; mixed lineage leukemia

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Authorship

JL designed, performed, and analyzed the majority of experiments and prepared manuscript. MW performed experiments involving NOD/SCID mice and FACS. JS assisted with sample preparation and PCR. CR, head of the laboratory, made contributions in overall experimental design, data evaluation, and manuscript preparation.

The mixed lineage leukemia (*MLL*) gene located on long arm of chromosome 11 (band 11q23) encodes a transcriptional regulator of *HOX* gene expression important in embryogenesis and hematopoiesis (1, 2). Numerous chromosomal translocations and rearrangements initiating within *MLL* reveal the recombinogenic nature of the locus and suggest that gain of *MLL* function contributes to the critical leukemogenic lesion (3). *MLL* alterations are involved in the development of a variety of hematological disorders including AML, ALL and MDS (4). Despite phenotypic heterogeneity *MLL*-rearranged acute leukemias share some clinical characteristics. This includes an aggressive course, early relapse after chemotherapy, as well as co-expression of lymphoid and myeloid antigens (5, 6) leading to the term 'mixed lineage leukemia' (7). Recently, this class of leukemias was shown to exhibit a specific gene expression profile distinct from other acute leukemias and consistent with an early hematopoietic progenitor (8). This and other studies (9, 10) provide evidence that *MLL* damage and rearrangement, and thus disease itself, initiate within an undifferentiated hematopoietic stem cell (HSC) compartment.

HSC are a rare cell population among hematopoietic progenitors present in bone marrow (BM). These cells are distinguished by self-renewal, long-term multi-lineage repopulating potential, and ability to reconstitute hematopoiesis both *in vivo* in myeloablated hosts and *in vitro* on stromal cells (11). The CD34+ stem-cell-enriched cell population comprises approximately 3% of normal human BM and 0.3–0.5% of cord blood mononuclear cells and includes pluripotent and totipotent progenitors including HSC. HSC are restricted within the CD34+/CD38 cell population that constitutes less than 10% of BM CD34+ progenitors (12). Leukemic HSC display properties similar to normal HSC including self-renewal and potential for propagation of a leukemic clone (13, 14). Recent data show that rearrangement of the *MLL* locus can be sufficient to promote malignant transformation of normal blood progenitors to leukemic stem cells (15–17). *MLL* fusion proteins can endow myeloid progenitors with self-renewal capacity, thus promoting the transition to leukemic stem cells (18). Cancer stem cells may be a general paradigm for tumor initiation, and have been identified in multiple tumor types (19–22).

Clinical evidence shows a clear relation between prior exposure to topoisomerase II (topo II) poisons and subsequent development of therapy-related acute leukemia (t-AML or t-ALL) characterized by chromosomal rearrangements involving the *MLL* gene (23). AML is the most frequent secondary malignancy after treatment with topo II poisons and is characterized by *MLL* translocations in 80% of cases (23–27). Many *MLL* rearrangements isolated from patients with t-AML localize within a well-characterized 8.3 kb break-point cluster region (bcr) that contains putative topo II cleavage recognition sequences and repetitive elements (28–30). We previously reported stimulation of alterations to a 1.6 kb hotspot within the *MLL* bcr in primary human CD34+ cells after exposure to the etoposide (31). Our results imply that etoposide treatment of non-leukemic human cells is sufficient to immediately promote the initiation of specific rearrangements of *MLL* consistent with the full spectrum of oncogenic events identified in leukemic samples. It seems that the spectrum of repair products within *MLL* gene is broader than described in clinical literature because *MLL* rearrangements are detected only once they are leukemogenic or limited methods available in routine diagnostics. The majority of cells that contain rearrangements did not proliferate in culture. This suggested a reduced proliferative capacity of most CD34+ cells during normal differentiation, or a general requirement for additional cellular mutations to gain a proliferative advantage characteristic of transformation. These results did not distinguish whether the stable *MLL* rearrangements occurred within the HSC compartment or within more committed progenitors that are contained within the CD34+ subpopulation.

In this study we determined the impact of a single exposure to etoposide on the most primitive cells within the CD34+ cell population. Surviving fraction of etoposide-treated

CD34⁺ cells were analyzed by their potential to repopulate the BM of immune-deficient non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and also their ability of initiating hematopoiesis on stroma (long-term culture initiating cell [LTC-IC] assay). A significant proportion of etoposide-treated clones (11 of 99) arising from HSC primitive population harbored *MLL* rearrangements, indicating that primitive stem cells that retain their ability to proliferate and differentiate are highly susceptible to acquiring potentially oncogenic repair structures.

Materials and methods

CD34⁺ cell isolation and culture

CD34⁺ cells were isolated and exposed to etoposide as previously reported (27). Use of human cells was approved by the Columbia University Institutional Review Board (IRB#15183). CD34⁺ cells were isolated from human umbilical cord bloods that were obtained from Columbia University Medical Center or Narutowicz Memorial Hospital (Krakow, Poland) and either frozen down for later use or used directly for experiments. Isolated cells were exposed to 20–50 μM etoposide [Sigma-Aldrich (St Louis, MO, USA); 20 mM stock solution prepared in dimethylsulfoxide (DMSO)] for 1 h and recovered 2 d to allow clearance of apoptotic cells. After recovery, surviving fraction of etoposide-treated and untreated control cells were counted using Trypan Blue exclusion method. Equal numbers of surviving and untreated cells were seeded on supportive feeder layer for LTC-IC or NOD/SCID mouse injection.

Long-term culture initiating cell assay

Murine fibroblast cell line M2-10B4, kindly provided by StemCell Technologies (Vancouver, BC, Canada), was prepared according to manufacturer protocol (http://www.stemcell.com/technical/28412_ltc-ic_H.pdf) and plated in 24-well plates into confluence to support human myeloid LTC-IC. Etoposide-treated or control CD34⁺ cell aliquots were resuspended in LTC-IC medium (H5100 supplemented with 10^6 μM hydrocortisone; StemCell Technologies) and plated on M2-10B4 feeder cell layer after careful removal of DMEM medium. Three concentrations were prepared in triplicates for both untreated control and etoposide-treated cohorts (100 000:10 000:1000 cells/well). LTC-IC cultures were incubated at 37°C in humidified incubator with 5% of CO₂ for minimum 6 wk with weekly one-half media changes. In some cultures, cells were exposed to several doses of etoposide (0.01 μM during weekly media change). After 6 wk, cultures were harvested and content of each well was plated in methylcellulose media with cytokine-supporting myeloid lineage differentiation (Methocult, GF H4434; StemCell Technologies). After 14 d, all progeny of HSC were scored as colony-forming units (CFU).

Transplantation into NOD/SCID mice

NOD/SCID mice were purchased from Jackson Laboratories (Bar Harbor, Maine), bred and maintained in sterile housing in the Columbia University Animal Facility according to protocols approved by the Animal Care Committee of Columbia University. Mice of 6–8-wk-old were irradiated (350 cGy from ¹³⁷Cs) 6 h before injection via tail vein. Each cell inoculum ranged from 3×10^5 to 1.6×10^6 cells. Additionally, one unpaired mouse was injected with 5×10^6 untreated control cells to provide enough engraftment for analysis of untreated control cells. After injections for 9 wk, mice were sacrificed, and BM isolated from four long bones of the limbs by flushing them with phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS). Engraftment was assessed by methylcellulose colony assays with hematopoietic growth factors supporting growth of only human progenitors (Methocult, GF H4434; StemCell Technologies). Human colonies representing progeny of primitive HSC were scored after 14 d.

Inverse polymerase chain reaction

Individual colonies were isolated from methylcellulose. DNA from almost 300 single colonies was amplified using multiple displacement amplification (MDA) kit (RepliGen kit; Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. MDA samples were initially screened for the quality of DNA with polymerase chain reaction (PCR) for housekeeping genes. Only clones with sufficient quality DNA were used for further DNA processing and inverse PCR (IPCR) analysis (that showed clear PCR bands with housekeeping gene β -actin). Of these, 159 clones were included into final statistical analysis after further verification (as described below). MDA samples were digested with *Xba*I restriction enzyme, circularized and IPCR was performed to analyze *MLL* gene stability within a 1.8 kb 'hot spot' region as previously described (Fig. 1A) (27). IPCR analysis was performed at least three times for each clone and only those samples that showed repeatedly by IPCR aberrant product were included in final analysis. Resultant cloned PCR products were sequenced with M13 forward and reverse primers. Nucleotide sequences were compared with *MLL* and analyzed using the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST, NCBI, Bethesda, MD, USA). All isolated aberrant ICPR products were screened for presence of *Xba*I recognition site at break-point junction and only those clones that contained novel sequence at their break points were taken into account.

Verification of aberrant inverse polymerase chain reaction products with sequence-specific primers

All sequences of aberrant clones were verified using sequence-specific primers. Duplications were verified by applying the IPCR primers (MF1 and MR1 followed by MF2 and MR2) but to unprocessed MDA sample instead of the ligation mix template. Two sets of nested primers were used to amplify each rearranged *MLL* product to ensure specificity of the product. Presence of aberrant product in unprocessed DNA was evidenced by the same size of PCR bands as in IPCR band obtained from *Xba*I digested and circularized DNA samples. Additionally, clones bearing *MLL* sequences from outside of 8.3 kb bcr fragment (inversions, insertions) were confirmed using sequence-specific primers that were designed to anneal to the 3' end of the clone sequence that stretched beyond *MLL* bcr that included: MF2 and 13.4R1 (ACC TAT GTA GCC ACC ACT CGG) together with 13.4F2 (ATG CTG CCT GCA CTG CAC TCC) and 13.4R2 (AAA CTA GCA ACC CAC AAG GG) as second round PCR for clone 13.4 and 17.2; MF2 and 41.3R (TTG CTA CCC AAA CAT TTT CTT TC) together with 41.3F2 (TCT CCT CCA TGC GAA TTT TT) and 41.3R for clone 41.3; MF2 and 8.9R1 (TTA CTC ATG AAT CAA TCC ATT CTT TT) together with 8.9F2 (GCC TGC ACT GCA CTC CTA AT) and 8.9R2 (AAT AAA CAT TGT ACA TAT TCA TGG GG) for clone 8.9. PCR reactions were run using puRe-Taq Ready-to-go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) according to previously described by the conditions (31).

All subsequent PCR products were compared in size to the original IPCR products and some of them were cloned into the pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

Results

Etoposide exposure stimulates hematopoietic stem cell proliferation

Previously, we reported an *in vitro* culture system and IPCR that allows characterization of the spectrum of stable, and potentially leukemogenic, genomic rearrangements induced by etoposide in CD34+ cells within a therapy-related translocation hotspot of the *MLL* bcr (31). We extended our experimental system to characterize the impact of etoposide on HSC which

represent only minor portion of the CD34+ cell population. Primary human CD34+ cells were isolated, exposed to 20–50 μM etoposide for 1 h, and recovered 2 d to allow clearance of apoptotic cells according to protocol previously described (31). After recovery, equal numbers of surviving etoposide-treated or untreated control cells were seeded on supportive feeder layer for LTC-IC or injected via tail vein into irradiated NOD/SCID mouse recipients. Six-week LTC-IC cultures were concluded with CFU colony formation in methylcellulose allowing for analysis of individual repair clones at the molecular level to determine the fidelity of repair at the *MLL* locus in the HSC compartment. In order to perform similar clonal analysis on HSC expanded in NOD/SCID recipients, BM was harvested after 12–14 wk *in vivo* and seeded into methylcellulose for human CFU colony formation. The number of CD34+ cells used for injection into NOD/SCID mouse was not sufficient to allow analysis of engraftment by cytofluorometry.

We analyzed the number of CFU derived from HSC surviving etoposide exposure as compared to untreated controls. LTC-IC cultures initiated by serial dilution seeding of either etoposide-treated or untreated primary human CD34+ cells onto feeder layers resulted in clonogenic cell output linearly related to the input inoculum (data not shown). Results from each of three independent experiments displayed some variation in clonogenic output that might be attributed to different sources of individual CD34+ cell batches, possible variation in freezing procedures between hospitals as well as different cycles of freezing and thawing. However, each experiment showed a significant increase in the number of LTC-ICs derived from surviving fraction of etoposide-treated HSC in comparison to untreated control (Table 1). Overall, untreated controls contained an average of 38.6 LTC-IC per 10^5 cells. A single exposure to 20 μM etoposide increased the number of LTC-IC to 117.7 per 10^5 cells, 151% stimulation. To better replicate clinical chemotherapy protocols that typically include several repeated cycles of etoposide administration, a parallel cohort of cells was exposed to several doses of etoposide during culture (0.01 μM added each week during media change). Multiple exposures to etoposide resulted in a significantly higher increase in LTC-IC to 357.7 per 10^5 cells, a 551% stimulation over untreated controls. The median number of CFU observed in LTC-IC cultures from etoposide-treated cells was significantly higher than untreated cells at all dilutions examined (P -value < 0.05; ANOVA software).

The difference was more dramatic in samples derived from NOD-SCID BM engrafted with etoposide-treated primary human CD34+ cells and harvested after 12–14 wk *in vivo* for CFU analysis (Table 2). In three independent experiments, human CFU were obtained from BM of mice engrafted with etoposide-treated CD34+ cells (57, 8 and 6 clones). By contrast, no CFUs were obtained from BM of mice engrafted with untreated cells (P < 0.01) within the cell inoculum that ranged from 3×10^5 to 1.6×10^6 cells. A total of 101 CFUs were obtained from untreated control CD34+ cells with inoculums of 5×10^6 cells to use as controls for sequence analysis.

Characterization of etoposide-induced *MLL* repair products

To determine the direct potential for etoposide to lead to *MLL* rearrangements and the stability of rearrangements in HSC, we used IPCR to determine the fidelity of repair in a 1.83 kb region in the 3' portion of *MLL* intron 11 that contains a translocation break-point hotspot in treatment-related leukemia and exon 12 that contains sequence homology to a putative topo II recognition sequence and is sensitive to DNaseI and multiple cytotoxic agents (30, 32, 33).

DNA was expanded from almost 300 single colonies using MDA system and quality of DNA determined by PCR of actin gene sequence. Only clones with sufficient quality DNA were used for further DNA processing and IPCR analysis (that showed clear PCR bands

with housekeeping gene β -actin). Of these, 159 clones were included into final statistical analysis after further verification (as described below).

IPCR of untreated samples gave almost exclusively a germline 1.83 kb product that was confirmed by sequencing (data not shown). By contrast IPCR of clones from etoposide-treated cells also revealed presence of variable-sized bands ranging from 250 bp to 1.2 kb representing alterations to the region.

Aberrant IPCR products were detected in 12 of 159 clones analyzed (99 etoposide-treated and 60 control CFUs; Table 3). Ten of these clones contained one altered IPCR product corresponding to the one of two *MLL* loci (alleles) present on two chromosomes. Two clones carried two unique aberrant IPCR products corresponding to both of the *MLL* loci (alleles) present on two chromosomes. Figure 1B presents 32 representative examples of etoposide-treated clones with five clones bearing altered IPCR products denoted by asterisks. We included into this final analysis only sequences that were subsequently verified for the presence of aberrant alleles in original, unprocessed MDA sample. Overall, 13 aberrant *MLL* alleles were found within 99 etoposide-treated clones in comparison to one aberrant *MLL* allele in 60 untreated control clones (13% vs. 1.6%; $P = 0.018$ by Fisher's exact test). This difference suggests that rearrangements are direct results of etoposide-induced DNA damage and aberrant repair event occurring in primitive HSC.

All 14 aberrant IPCR products were sequenced to characterize the *MLL* illegitimate repair events that occur during the normal process of DNA repair in HSC. We verified that all aberrant amplification products were not technical artifacts produced during restriction enzyme digestion and ligation. Presence of aberrant sequences were confirmed in original, unprocessed DNA by independent PCR reactions using *MLL* sequence-specific primers (duplications), or using sequence-specific primers annealing to foreign sequence identified at the 3' end of the clone (inversions, insertions). Figure 2 shows an example of three representative PCR reactions for detection of duplicated products both in unprocessed DNA and in digested and circularized samples.

Sequencing of all products revealed the fine structure of the break points and repair products (Fig. 3). All isolated aberrant ICPR products were confirmed not to bear *Xba*I recognition site at break-point junction and this was evidence that the fusion was not a technical artifact (created after digestion with *Xba*I and circularization) but generated during repair of broken DNA after exposure to genotoxic agent. Nine were partial tandem duplications (PTD), three were inversions (with two clones bearing the same event), and one fused 280 bp sequence from chromosome 8q23. That unique repair products were recovered from the majority of LTC-IC examined indicates that the observed stimulation of LTC-IC by etoposide exposure is not due to outgrowth of a single aberrant clone in each case. This spectrum of etoposide-induced *MLL* locus products identified in primitive HSC after LTC-IC or NOD/SCID mouse engraftment closely corresponded to our previous findings of aberrant repair events etoposide-treated CD34+ cells recovered after expanding them in liquid culture for 2 wk (31). Two independent etoposide-treated clones (13.4 and 17.2) obtained from the same LTC-IC culture contained the same rearrangement (inversion *MLL* bcr 5695/25707 $\rightarrow$ 24842). This could be due to expansion of one aberrant HSC. In addition, another two etoposide-treated colonies contained two different aberrant alleles apparently resulting from two separate illegitimate events leading to coexistence of two separate rearrangements in one cell. Alternatively, it is possible that repair events occurred in G2 or in two daughter cells following the first cell division in methylcellulose resulting in an apparent mixed colony. The single aberrant PCR product detected in a clone from untreated control cells was a PTD. Microhomologies of 1 to 13 bp were present in half of the break-point junctions (7 of 14). In addition, one junction demonstrated 58 bp of perfect alignment at the junction

indicating that single-strand annealing (SSA) is a joining mechanism used in primary HSC just as observed in several other experimental DNA break systems (34). Overall, 44% of break-point junctions localized within repetitive elements. Of the nine PTD, three break points consisted of ALU/ALU or LINE/LINE joints, and one consisted of a mixed ALU/LINE alignment.

Size of all aberrant IPCR products, as read from electrophoresis gel with IPCR and verifying PCR products, corresponded precisely to those calculated on the basis of the nucleotide sequence and primer location as described previously by us (Fig. 3) (31). Because of structure of duplicated alleles that include double sites for reverse primers, IPCR favored amplification of the shorter product (with reverse primer annealing site closer to break-point junction instead of further site, created during ligation process). Thus, amplification of duplicated alleles resulted in shorter IPCR amplicons than germline bands. The evaluation of the duplicated clone sizes was based on nucleotide calculations presented in Table 4.

The only clone that bore foreign sequence had three bases of homology within the junction but no flanking repetitive element sequence. Overall, the break-point junctions were similar to break points sequenced from leukemic cells (35). Several junctions localized to breakpoint junctions from CD34+ cells in our previous study and in material from patients with therapy-related leukemia (1, 35).

Discussion

The small HSC population is believed to be the target cell for leukemia-initiating *MLL* rearrangements (8). The difficulty in isolating and characterizing these cells has hampered efforts to determine the impact of etoposide on proliferative and developmental potential, as well as genetic stability, of the undifferentiated HSC compartment. Previously, we reported an *in vitro* culture system and IPCR that allows characterization of the spectrum of stable, and potentially leukemogenic, genomic rearrangements initiating in CD34+ cell population within a therapy-related translocation hotspot of the *MLL* bcr induced by etoposide (31). We extended our experimental system to characterize the impact of etoposide on HSC which represent only minor portion of the CD34+ cell population, using experimental conditions that selectively support their clonal expansion. Our results showed that CD34+ cells surviving treatment with physiological dose of etoposide are able to proliferate and differentiate, engraft and repopulate BM of NOD/SCID mice, as well as initiate hematopoiesis in LTC-IC.

MLL rearrangements, including inversions and foreign sequence fusions, are readily detectable within the surviving HSC after a sublethal etoposide exposure, indicating that stem cells are highly susceptible to acquiring potentially oncogenic repair structures. Our results are consistent with the development of *MLL*-rearranged therapy-related leukemias in a small fraction of patients receiving chemotherapy regimens that include topo II poisons (25, 26). Although it is difficult to estimate the frequency of etoposide-induced rearrangements in this population, we observed them at approximately 10-fold higher frequency than spontaneously arising aberrations analogous to *de novo* AML leukemias (duplications, inversions, foreign sequence fusions). Translocations involving the *MLL* locus are observed in 80% of ALL and 45% of AML cases of infant leukemias (<1 yr of age), and in 5% of AML and 10% of ALL cases of adult *de novo* and therapy-related leukemias, whereas the frequency of *MLL* translocations increases to more than 70% of those who previously received etoposide-containing regimens. Our results correlate with a higher incidence of *MLL* rearrangements occurring in patients treated with podophylotoxins as well as *in utero* exposure to environmental topo II inhibitors (36). The initiation of genome

rearrangements in the earliest stem cell compartment may be due to open chromatin structure, altered expression or function of DNA repair proteins.

Interestingly, two clones derived from etoposide-treated HSC from two separate experiments displayed the same duplication with an almost identical break-point junction (clones 8.3 and 19.2). This might suggest existence of ubiquitous pattern of genetic lesions appearing in individuals as a response to this genotoxic agent. Such lesions might be a common long-term side effect of topo II inhibitor exposure but without contributing to secondary leukemia. It seems that initial formation of *MLL* locus aberrations might be general phenomenon but individual genetic factors that affect DNA damage sensor and repair protein machinery might influence both the frequency and spectrum of repair products (37). Alternatively, aberrant illegitimate repair could initiate rearrangements in multiple cell types but expression of a fused protein product and its biological effect depend on the differentiation level of cells that acquire them. Additional factor might be their survival capacity that is highest in less differentiated cells and enable propagation of the genetic defect (38).

The LTC-IC conditions used here are known to promote development and terminal differentiation of primitive myeloid progenitors. Although the NOD/SCID assay can support proliferation and differentiation of HSC into both myeloid and lymphoid cells (39, 40), BM harvested from NOD/SCID transplant recipients was plated into methylcellulose assays that favor differentiation of myeloid progenitors. The high frequency of PTDs detected in this experimental model seems to be consistent with frequent clinical association of PTDs with myeloid leukemia. This may reflect a common repair mechanism used within the myeloid compartment (41, 42). It is possible that culture conditions that favor other than myeloid differentiation might provide different spectrum of aberrant products. Further studies on myeloid–lymphoid LTC-IC (ML-LTC-IC) assay might provide insight (43–45). PTDs in AML are associated with duplications encompassing several exons and resulting in *MLL*–*MLL* fusion transcripts that contribute to leukemogenesis by recessive gain-of-function mechanisms (41, 46). We detected only short *MLL* duplications either because these are physiologically favored or because our IPCR method did not detect longer PTD tracts. Similar *MLL* PTD were also reported in healthy donor blood mononuclear cells as well as in CD34+enriched cell fraction of cord blood mononuclear cells (47, 48). Although PTD in AML is associated with poor prognosis, our data suggest that the majority of *MLL* duplications are not involved directly in malignant transformation. This might suggest also requirement of additional oncogenic mutations necessary to establish the malignant phenotype.

This study revealed presence of inversions among etoposide-induced lesions that in some cases included *MLL* sequence outside of the 8.3 kb bcr. Interestingly, etoposide promoted inversions were not detected in the bulk CD34+ population immediately following etoposide treatment, but rather are restricted to CD34+ cells after 2 wk in culture (31), as shown previously, or to HSC-derived progenitors as shown here. This suggests a distinct set of repair mechanisms is used by early pluripotent cells capable of long-term proliferation and differentiation. Inversions of *MLL* are rarely associated with t-AML with only a single case reported in the literature (49). In this case the long latency (6 yr persistence of a clone with inv 11 in the bone marrow before development of t-AML) suggests a benign character of this genetic lesion and requirement of other collaborating mutations (49). Several studies have suggested that *de novo* ALL cases with inversions affecting the 11q23 region represent clinically and biologically different entities generally presenting favorable clinical outcome as compared with those defined by 11q23 translocations (4, 50, 51).

It is significant that the HSC compartment not only survives following 1 h of etoposide treatment but also gains proliferative potential, including those cells that acquire potentially oncogenic *MLL* 11q23 rearrangements. This was observed in all samples and also by two different methods of assaying the most primitive stem cell compartment – *in vitro* LTC-IC and *in vivo* NOD/SCID mice. Individual colonies derived from etoposide-treated cells were capable of several fold expansion following harvesting (Libura and Richardson, unpublished). However, we do not attribute this observation to the immortalization of a small number of CD34+ cells. Individual clone analysis by IPCR produced a large number of unique *MLL* rearrangements in these clones, indicating that they mainly derived from multiple initiating and proliferating stem cells rather than outgrowth of a single immortal clone. Etoposide may eradicate more differentiated highly proliferating CD34+ cells while sparing less mature and more primitive cells. Etoposide may also stimulate intrinsic cellular programs within this early cell population that lead to proliferation and expansion. G2/M cell cycle arrest and inhibition of proliferation of etoposide-treated cells for several days before seeding CD34+ cells on feeders may result in an increased life span and delayed differentiation in comparison to continuously cycling untreated control cells that would decline in number sooner (34, 52). Proliferative potential was further increased after multiple, non-cytotoxic doses of etoposide analogous to clinical protocols that include multiple cycles of chemotherapy. In additional experiment, we seeded the same numbers of untreated cells on feeder layers and treated some wells with non-lethal doses of etoposide (0.01 μM /wk). The results paralleled our so far observations (411% stimulation of colony counts, unpublished data) and confirmed the hypothesis that non-cytotoxic doses of etoposide put on hold the less primitive progenitors by blocking the progress through cell cycle checkpoints and thus delay the proliferation and terminal differentiation of these progenitors resulting in higher CFU readouts. The CD34+ cells used by us were derived from umbilical cord blood. According to our observations and some other data, this population has been shown to possess greater long-term proliferative potential than adult BM-derived CD34+ cells (45). Whether similar results would be obtained from adult BM is not clear. The cellular response of this population to etoposide may further augment this proliferative potential by producing a surviving fraction of cells which are particularly sensitive to cytokines present in the culture conditions.

Our results suggest that the earliest stem cell compartment is particularly permissive for both the initiation of genome rearrangements and the survival and stability of cells that acquire them. These findings indicate that anti-tumor targeting strategies will need to focus on this primitive and somewhat elusive population.

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Figure 1. IPCR analysis to detect etoposide-induced rearrangements initiating within *MLL* bcr. (A) Schematic representation of the *MLL* gene locus on chromosome band 11q23. The 8.3 kb *MLL* bcr, flanked by *Bam*HI sites, includes exons 8–14 and intervening introns. *Xba*I sites are 2.6 kb apart within the *MLL* bcr. Genomic DNA was digested with *Xba*I and circularized. Nested PCR reactions were carried out with F1–R1 primer pair, followed by F2–R2 primer pair. BH – *Bam*HI; X – *Xba*I; MF1, MF2, MR1, MR2 – primers used for PCR (see ‘Materials and methods’ section in previous report (31)). (B) Representative IPCR products. Lanes 1–32: Etoposide-treated samples with most cases that showed the expected 1.8 kb germline product and five clones that give alternative products representing possible rearrangements of *MLL* (sample # 7, 11, 18, 19, 27). Asterisk (*) – aberrant products.

Figure 2.

Verification of aberrant IPCR products with sequence-specific primers and unprocessed DNA: example of three representative PCR reactions for detection of duplicated and inverted products both in unprocessed and in digested and circularized DNA. (A) Duplications were verified by applying the IPCR primers but to unprocessed MDA sample instead of the ligation mix template and the presence of aberrant product in unprocessed DNA were evidenced by the same size of PCR bands as in IPCR (see description in 'Materials and methods' section). Example of verification of three duplications using unprocessed MDA samples, ligation mix, control MDA samples and sequencing plasmids: asterisk (*) – aberrant products; M – marker; clone 15.2: 1 – MDA sample, 2 – sample ligation mix, 3 – control MDA sample, 4 – control genomic DNA, 5 – sequencing plasmid (positive control); clone 27.1: 6 – MDA sample, 7 – sample ligation mix, 8 – control MDA sample, 9 – control genomic DNA, 10 – sequencing plasmid (positive control); clone 43.1: 11 – MDA sample, 12 – sample ligation mix, 13 – control MDA sample, 14 – control genomic DNA, 15 – sequencing plasmid (positive control). (B) Verification of clone 41.3 containing inverted sequence from outside *MLL* bcr was performed with following primers MF2 and 41.3R (TTG CTA CCC AAA CAT TTT CTT TC) together with 41.3F2 (TCT CCT CCA TGC GAA TTT TT) and 41.3R in second round PCR that resulted in final PCR product as long as 171 bp: 1 – sample MDA, 2 – sample ligation mix, 3 – control MDA, 4 and 5 – control genomic DNA, 6 – sequencing plasmid (positive control). Ma – X174 *Hae*III digest; Mb – Lambda *Bst*EII digest markers (NEB, Beverly, MA).

Experiment Series	Clone name	IPCR product length	Repetitive element localization	Type of aberration	Breakpoint Junction sequence aligned against parental sequences
LTC-IC 1	12.1	861 bp = (6183-5531) + (4675-4471) = 652+209	ALU/LINE	Duplication MLL bcr 6183/4471	MLL bcr 6183 bp GCTAACACAGTGAACCCCATCTCTACTAAAAACAAAAATAGCC GCTAACACAGTGAACCCCATCTAAACAAATCTGGAAGGATTCACACCA GTGTTTTAAAGTATATATCTAGAAAAACATCTGGAAGGATTCACACCA MLL bcr 4471 bp
NOD/SCID	25.3	262 bp = (5589-5531) + (4675-4471) = 58 + 204	(-)LINE	Duplication MLL bcr 5589/4471	MLL bcr 5589 bp ATGACCAAGTCTGTGATAAATCTCTCCAAACAAATCTGGAAGGATTCACAC ATGACCAAGTCTGTGATAAATCTCTCCAAACAAATCTGGAAGGATTCACAC AAAGTGTGTTTTAAAGTATATATCTAGAAAAACATCTGGAAGGATTCACAC MLL bcr 4471 bp
NOD/SCID	25.1	684 bp = (6161-5531) + (4675-4621) = 630 + 54	ALU/(-)	Duplication MLL bcr 6161/4621	MLL bcr 6161 bp AAGGTCAGGAGATTGAGACCATCTCGCTAACACAGTGAACCCCATCT AAGGTCAGGAGATTGAGACCATCTCAATTTAAATAGGAAGCAGTAGT TACAATGAGGAAGGAAAAAGTAGCACAAATTTAAATAGGAAGCAGTAGT MLL bcr 4621 bp
LTC-IC 2	8.3	682 bp = (6022-5531) + (4675-4484) = 491 + 191	(-)LINE	Duplication MLL bcr 6022/4484	MLL bcr 6022 bp CTGGATTATCCTAGTTAGTATTTTGAATAATCTTATATCAATATGAAAAATA CTGGATTATCCTAGTTAGTATTTTGAATAATCTTATATCAATATGAAAAATA TATATCTAGAAAAACATCTGGAAGGATTCACACAAATATTAAGAGTGT MLL bcr 4484 bp
LTC-IC 2	43.1	1292 bp = (6351-5531) + (4675-4203) = 820 + 472	ALU/ALU	Duplication MLL bcr 6351/4203	MLL bcr 6351 bp TTGCAGTGAGTCGAGATCGCAC (37 BP) CTCAAAAATAAATAAATAA TTGCAGTGAGTCGAGATCGCAC (37 BP) CTCAAAAATAAATAAATAA TTGCAGTGAGTCGAGATCGCAC (37 BP) CTCAAAAATAAATAAATAA MLL bcr 4203 bp
LTC-IC 3	15.2	627 bp = (6101-5531) + (4675-4618) = 570 + 57	ALU/(-)	Duplication MLL bcr 6101/4618	MLL bcr 6101 bp GGGCGTGGTGGCTCAGCGCTATAATCTCTTACAGTTCTTAATCTCT GGGCGTGGTGGCTCAGCGCTATAATCTCTTACAGTTCTTAATCTCT TTGTCATTATAAGGACATATCTACATCTTACAGTTCTTAATCTCT MLL bcr 4618 bp
LTC-IC 3	13.2	810 bp = (5577-5531) + (4675-3910) = 45 + 765	(-) / LINE	Duplication MLL bcr 5577/3910	MLL bcr 5577 bp ACTCCTAAAGCATGACCAAGTCTGTGATAAATCTCTCCATGCGAATTT ACTCCTAAAGCATGACCAAGTCTGTGATAAATCTCTCCATGCGAATTT CAATATTGCTCATAATAAATAAAGTGAACCAACCGAAAGTCCATC MLL bcr 3910 bp
LTC-IC 3	19.2	675 bp = (6022-5531) + (4675-4491) = 491 + 184	(-) / LINE	Duplication MLL bcr 6022/4491	MLL bcr 6022 bp CTGGATTATCCTAGTTAGTATTTTGAATAATCTTATATCAATATGAAAAATA CTGGATTATCCTAGTTAGTATTTTGAATAATCTTATATCAATATGAAAAATA ATATCTAGAAAAACATCTGGAAGGATTCACACAAATATTAAGAGTGTG MLL bcr 4491 bp
LTC-IC 3	27.1	519 bp = (5967-5531) + (4675-4592) = 436 + 83	LINE / LINE	Duplication MLL bcr 5967/4592	MLL bcr 5967 bp CTTATTTAGTTTGAACCAATGTGCTCAATAATCTTATGGAAGAAAGAA CTTATTTAGTTTGAACCAATGTGCTCAATAATCTTATGGAAGAAAGAA TTCTGAGTTTTTTGGAGTATGTACACCTTTACAATGAGGAAGGAAAAAG MLL bcr 4592 bp
LTC-IC 2 controls	22.2	474 bp = (5823-5531) + (4675-4493) = 292 + 182	LINE / LINE	Duplication MLL bcr 5823/4493	MLL bcr 5823 bp CAAGTTGGAGACATAAACCATTTTACCTCAATAATTTAGTGTATATTT CAAGTTGGAGACATAAACCATTTTACCAAAATATTAAGAGTGTGTTGGA GAAAAACATCTGGAAGGATTCACACCAAAATATTAAGAGTGTGTTGGA MLL bcr 4493 bp
LTC-IC 2	8.9	523 bp = (5565-5531) + (85342-85053) + (4675-4466) = 34+280+209	(-) / (-)	fusion MLL bcr 5565/85053 → 85342 clone KB126H7 chr 8q23	MLL bcr 5561 bp GCATGCTGCTGCACACTGCACTCTAAAGCATGACCAAGTCTGTGATAAAT GCATGCTGCTGCACACTGCACTCTAAAGCATGACCAAGTCTGTGATAAAT AGAAATATGATAAATGTTTAAATCATATAAATGAGAAATACAAAGAAATA clone KB126H7, chromosome 8q23
LTC-IC 2	41.3	841 bp = (5638-5531) + (8824-8299) + (4675-4466) = 107+525+209	LINE / (-)	Inversion MLL bcr 5638/8824 → 8299	MLL bcr 5638 bp TTATGTTGACATGATTTGACATCTACAAAAAACTATGAGTTGACAGAGA TTATGTTGACATGATTTGACATCTTAAACAGTGAACCTTATATGATTA TCTTCAAACTGATCTGCTTATCTTACTTAAACAGTGAACCTTATATGATTA MLL bcr 8824 bp
LTC-IC 2	13.4	1238 bp = (5695-5531) + (25707-24842) + (4675-4466) = 164+865+209	LINE / (-)	Inversion MLL bcr 5695/25707 → 24842	MLL bcr 5695 bp TAAGTACCCCTCACCAAAATCCCTAAGTGTTAATATGTTCTGTGTGT TAAGTACCCCTCACCAAAATCCCTAAGTGTTAATATGTTCTGTGTGT GTCTATAAGCCAGCAAACTGACCTAAGTGTATAAAGGTTAGAATAATGG MLL 25707 bp
LTC-IC 2	17.2	1238 bp = (5695-5531) + (25707-24842) + (4675-4466) = 164+865+209	LINE / (-)	Inversion MLL bcr 5695/25707 → 24842	MLL bcr 5695 bp TAAGTACCCCTCACCAAAATCCCTAAGTGTTAATATGTTCTGTGTGT TAAGTACCCCTCACCAAAATCCCTAAGTGTTAATATGTTCTGTGTGT GTCTATAAGCCAGCAAACTGACCTAAGTGTATAAAGGTTAGAATAATGG MLL 25707 bp

Figure 3.

Spectrum of repair products and overlapping homology of representative etoposide-induced break-point sequence junctions. Red indicates sequence 3' of the break-point junction and blue indicates sequence 5' of the break-point junction. 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 insertions or homologies.

Table 1

Colony-forming units in LTC-IC from etoposide-treated and control untreated CD34+ cells

	No. of CD34+ cells seeded per well	No. of CFU colonies ¹		
		Untreated control	+Etoposide (1 h 20 μ M)	+Etoposide (1 h 20 μ M)+ ($\times$ 0.01 μ M/wk)
I	10 ⁵	31.9 ($\pm$ 6.8)	50.6 ($\pm$ 17.3)	51.2 ($\pm$ 17.7)
	10 ⁴	2.35 ($\pm$ 0.2)	9.37 ($\pm$ 2.8)	19.2 ($\pm$ 9)
	10 ³	0	0.75 ($\pm$ 0.65)	3.52 ($\pm$ 1.35)
	Average per 1 $\times$ 10 ⁵	27.4	73.1	198.4
	% Stimulation ²	–	167%	624%
II	10 ⁵	1.5 ($\pm$ 1.9)	2.5 ($\pm$ 1.7)	4.3 ($\pm$ 2)
	10 ⁴	0	0	4.66 ($\pm$ 1.25)
	10 ³	0	0	0.5 ($\pm$ 1)
	Average per 1 $\times$ 10 ⁵	1.5	2.5	3.4
	% Stimulation	–	66%	127%
III	10 ⁵	96.0 ($\pm$ 55.6)	153 ($\pm$ 88.2)	413 ($\pm$ 38)
	10 ⁴	7.8 ($\pm$ 7.2)	40.25 ($\pm$ 24.9)	133 ($\pm$ 15.5)
	10 ³	nd	nd	nd
	Average per 1 $\times$ 10 ⁵	87.0	277.7	871.5
	% Stimulation	–	219%	902%
Average per 1 $\times$ 10 ⁵ cells	38.6	117.7	357.7	
Average % stimulation	–	151%	551%	

¹ Number of CFU colonies obtained in LTC-IC assays derived from primary human CD34+ cells are average values from three or more replicates for all experimental conditions at each cell dilution with standard deviations.

² % stimulation represents the percentage increase of LTC-IC average per 10⁵ cells in each etoposide-treated population. This number was obtained by calculating percentage of cells in etoposide-treated populations in relation to controls to reveal the percentage of growth stimulation in relation to control. This was calculated as [(column 2-column 1)/column 1] expressed as a percentage value showing the percentage of growth stimulation in relation to control.

Table 2

Colony-forming units derived from NOD/SCID mice engrafted with etoposide-treated and control untreated CD34+ cells

No. of CD34+ cells per injection	No. of CFU ^I		<i>P</i>
	Untreated	+Etoposide (1 h 20 μM)	
6.0×10^5	0	6	
3.0×10^5	0	8	
1.5×10^6	0	57	
Total	0	71	<0.01 ²

^I *P*-value calculated by the *t*-test.

² Results are from three separate injections with control untreated and etoposide-treated CD34+ cells.

Table 3

Frequency of aberrant IPCR products obtained from etoposide-treated and untreated control colony-forming units

	No. of aberrant alleles/total no. of CFU analyzed		<i>P</i>
	Untreated	+Etoposide	
Total	1/60	13/99	< 0.018 ^{<i>I</i>}
LTC-IC	1/40	10/75	
NOD/SCID	0/20	3/24	

^{*I*} *P*-values calculated by the Fisher's two-tailed exact test.

Table 4

Nucleotide calculation of the duplicated clone sizes on the basis of the nucleotide sequence and IPCR (MF2 and MR2) primer location within the *MLL* gene [see 'Materials and methods' section in previous report (31)]

Clone 12.2	Shorter: $(6183 - 5531) + (4675 - 4471) = 861$ bp Longer: $(6183 - 5531) + (7148 - 4471) + (4675 - 4466) = 3538$ bp
Clone 25.3	Shorter: $(5589 - 5531) + (4675 - 4471) = 262$ bp Longer: $(5589 - 5531) + (7148 - 4471) + (4675 - 4466) = 2944$ bp
Clone 25.1	Shorter: $(6161 - 5531) + (4675 - 4621) = 684$ bp Longer: $(6161 - 5531) + (7148 - 4621) + (4675 - 4466) = 3366$ bp
Clone 8.3	Shorter: $(6022 - 5531) + (4675 - 4484) = 682$ bp Longer: $(6022 - 5531) + (7148 - 4484) + (4675 - 4466) = 3364$ bp
Clone 43.1	Shorter: $(6351 - 5531) + (4675 - 4203) = 1292$ bp Longer: $(6351 - 5531) + (7148 - 4203) + (4675 - 4466) = 3974$ bp
Clone 15.2	Shorter: $(6101 - 5531) + (4675 - 4618) = 627$ bp Longer: $(6101 - 5531) + (7148 - 4618) + (4675 - 4466) = 3309$ bp
Clone 13.2	Shorter: $(5577 - 5531) + (4675 - 3910) = 810$ bp Longer: $(5577 - 5531) + (7148 - 3910) + (4675 - 4466) = 3492$ bp
Clone 19.2	Shorter: $(6022 - 5531) + (4675 - 4491) = 675$ bp Longer: $(6022 - 5531) + (7148 - 4491) + (4675 - 4466) = 3357$ bp
Clone 27.1	Shorter: $(5967 - 5531) + (4675 - 4592) = 519$ bp Longer: $(5967 - 5531) + (7148 - 4592) + (4675 - 4466) = 3201$ bp
Clone 22.2	Shorter: $(5823 - 5531) + (4675 - 4493) = 474$ bp Longer: $(5823 - 5531) + (7148 - 4493) + (4675 - 4466) = 3156$ bp