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In Vitro Cleavage of the *MLL* Gene by Topoisomerase II Inhibitor (Etoposide) in Normal Cord and Peripheral Blood Mononuclear Cells

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Abstract

The correlation between infant leukemia and in utero exposure to topoisomerase II (topo-II) inhibitor has been clarified. We examined the in vitro effect of topo-II inhibitor (etoposide) on cleavage of the *MLL* gene in cord and peripheral blood mononuclear cells (MNCs). Southern blot analysis showed cleavage of the *MLL* gene in peripheral blood MNCs of infants when the MNCs were exposed to etoposide. MNCs were incubated with etoposide at various concentrations (1 to 50 μM), and a ligation-mediated polymerase chain reaction (LM-PCR) was used to detect double strand breaks (DSBs) of DNA in intron 8 of the *MLL* breakpoint cluster region. PCR products obtained with LM-PCR were subcloned and sequenced to identify the breakpoint in the *MLL* gene. The PCR products indicated DSBs of the *MLL* gene were obtained without any difference in the incidence between 3 different samples (cord and peripheral blood from infants and children). Sequencing analysis showed that the DSBs occurred on the telomeric side of intron 8 and near exon 9. There was no evidence that the cord blood was more susceptible to *MLL* DNA breakage by topo-II inhibitor than were other cells. Instability of the partner gene during the fetal period could be associated with the pathogenesis of infant leukemia. *Int J Hematol.* 2002;76:74-79.

Key words: Hematopoietic cells; *MLL* gene; Topoisomerase II inhibitor; Ligation-mediated PCR; Double strand break

1. Introduction

Infant acute leukemia, which is characterized by a high white blood cell count, organomegaly, and a high prevalence of extramedullary involvement, frequently involves chromosome 11q23 translocations [1-3]. The gene on chromosome 11q23 that is disrupted by these translocations has been designated *MLL* and also is associated with therapy-related acute leukemia in patients who have received topoisomerase II (topo-II) inhibitors for previous neoplasms [4-6]. Twins with infant leukemia have been shown to have identical cytogenesis and breakpoints of the *MLL* gene and have provided definitive evidence of intrauterine rearrangement of the *MLL* gene [7,8]. Exposure in utero to some drugs and foods that have functional similarity to topo-II inhibitors and to certain environmental factors has been suggested to affect rearrangement of the *MLL* gene [9,10]. These findings are supported by results of in vitro studies showing that rearrangement of the *MLL* gene is induced by topo-II inhibitors in human hematopoietic cells [11,12].

In light of these findings, we hypothesized that cord blood is more susceptible than is other blood to site-specific cleavage of the *MLL* gene by topo-II inhibitors. Therefore we analyzed in vitro cleavage of the *MLL* gene in different

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hematopoietic samples. Although a low concentration of etoposide also induced gene cleavage, there was no difference between the incidence of cleavage in cord blood samples and that in peripheral blood samples.

2. Materials and Methods

2.1. Peripheral Blood and Cord Blood Samples

Peripheral blood samples were obtained from healthy infants and children after informed consent was obtained from their parents. All infants and children had no history of hematopoietic malignant disease or administration of any chemotherapeutic or immunosuppressive agent. Cord blood samples were collected at the birth of term newborns after informed consent was obtained from their mothers.

2.2. In Vitro Stimulation of Mononuclear Cells by Topo-II Inhibitors

As primary hematopoietic cells, mononuclear cells (MNCs) were separated from cord and peripheral blood samples, resuspended in RPMI 1640 with 10% fetal calf serum at a concentration of 1×10^6 cells/mL, and incubated for 36 hours in the presence of 1% phytohemagglutinin. MNCs were then coincubated with etoposide at various concentrations (0, 1, 10, and 50 μM) for 16 hours according to previously described methods [11]. DNA was extracted from the MNCs for further analysis.

2.3. Southern Blot Analysis of the MLL Gene

Southern blot analysis was performed to examine whether cleavage of the *MLL* gene could be induced by etoposide. Cord and peripheral blood MNCs were stimulated with 50 μM etoposide for 16 hours. Extracted DNA was digested with *Bam* HI, then electrophoresed and transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK). The transferred membrane was hybridized with an 880–base-pair (bp) *MLL* complementary DNA probe covering the breakpoint cluster region (BCR) of the *MLL* gene, which was provided by Prof. M. Greaves (Leukaemia Research Fund Centre, London, UK). Other restriction enzymes were not used to detect gene cleavage, because only a small volume of DNA sample was available in this study.

2.4. Ligation-Mediated Polymerase Chain Reaction

To detect double strand breaks (DSBs) in DNA, ligation-mediated polymerase chain reaction (LM-PCR) was performed as described previously [13-15]. The BW linker was produced by annealing the BW-1 and BW-2 oligonucleotides, which have been described previously [16]. Briefly, 2 μg of DNA was ligated to the BW linker with T4 DNA ligase (Takara Shuzo, Otsu, Japan), precipitated with ethanol, and dissolved in 20 μL of distilled water. One microliter of the ligated sample was analyzed by means of PCR with Ex Taq (Takara) with BW-1 linker primer (0.02 μM) and specific primers (0.4 μM) of the *MLL* gene located in intron 8, because most of the cleavage of the *MLL* gene occurs on the telomeric side of intron 8 or near exon 9 [11,17-20]. Semi-nested PCR was performed with a specific primer (MLL-BCR 5492f, 5′-ACATAGCAATCTCACAGGGTTCC-3′) and BW-1 primer (5′-GCGGTGACCCGGGAGATCTGA ATTC-3′) for the first round of PCR and with an internal specific primer (MLL-BCR-5676f, 5′-CCTCACCCAAAT TCCCTAAGTGT-3′) and BW-1 for the second round. The conditions for PCR were as follows: 94°C for 2 minutes followed by 30 cycles of 94°C for 1 minute, 65°C for the first round and 57°C for the second round for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. For detection of the PCR products showing DSBs, each sample was electrophoresed and visualized with ethidium bromide. PCR products were then purified with a PCR purification kit (Qiagen, Santa Clarita, CA, USA) and subcloned into pCRII vector (Invitrogen, Tokyo, Japan) with a TA-cloning kit (Promega, Madison, WI, USA). Plasmid DNA was extracted with the Qiaprep Spin Plasmid system (Qiagen), and insert DNAs were sequenced with M13 forward and reverse primers with a 373A Autosequencer (Applied Biosystems, Urayasu, Japan). The nucleotide sequences obtained were compared with the sequences of intron 8 of the *MLL* gene.

3. Results

The results of Southern blot analysis are shown in Figure 1. The germline *MLL* fragment (8.3 kilobases [kb]) was seen in all samples. One of the *MLL* fragments induced by the treatment with etoposide (approximately 1.5 to 1.6 kb) was detected in 2 of the 3 samples of peripheral blood MNCs of infants, whereas no fragment induced by etoposide was observed in either cord blood (3 samples) or peripheral blood MNCs of children (3 samples). Another fragment (approximately 7 kb) may be hidden behind the germline band. According to these findings, only a small population of MNCs show *MLL* gene cleavage by topo-II inhibitors.

To clarify whether the DSBs of the *MLL* gene occur in the etoposide-treated MNCs, LM-PCR was performed. A total of 38 samples were analyzed in the study; 13 samples were from cord blood, 11 from peripheral blood of infants, and 14 from peripheral blood of children. The results of LM-PCR are given in Table 1, and the PCR results from representative samples are shown in Figure 2. Although the DSBs were not detected in control MNCs without stimulation, 1 to 50 μM of etoposide induced DSBs in both cord and peripheral blood MNCs (Figure 2). Some of the samples showed a clear single band (lanes 2, 4, 5, and 6), whereas others had several different bands (lanes 1, 3, and 7). These findings indicate that DSBs occurred at several sites in the *MLL* gene. However, there was no difference in the incidence of DBSs between the 3 different blood samples. A low concentration of etoposide (1 μM) also induced DSBs in MNCs, in accordance with the findings of a previous study (Table 1) [11].

Sequencing analysis also identified the DSB sites induced by etoposide. Subcloning and sequencing were tried on all samples that showed the PCR products, but findings were obtained in only 4 samples. Subcloning and sequencing were

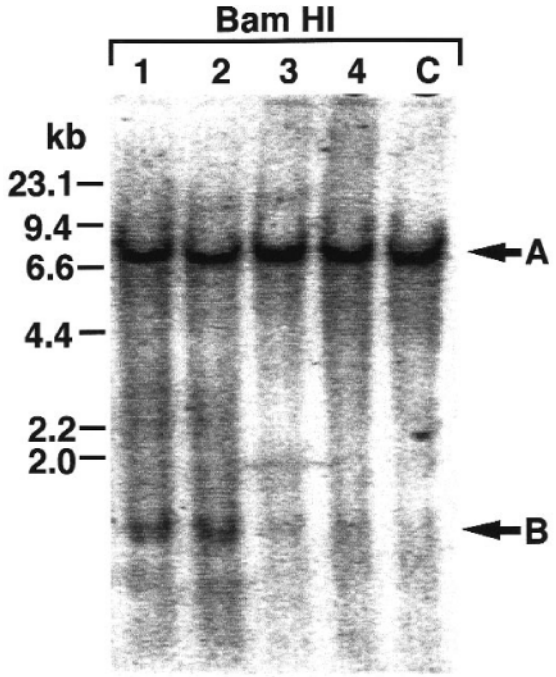

Figure 1. Detection of *MLL* gene cleavage by means of treatment with etoposide in normal mononuclear cells (MNCs). MNCs were treated with 50 μM etoposide for 16 hours, and extracted DNA was digested with *Bam*HI, electrophoresed, transferred to a nylon membrane, and hybridized with the *MLL* complementary DNA probe. In addition to the germline *MLL* fragment (approximately 8.3 kilobases [kb]) (arrow A), a fragment of the *MLL* gene induced by the treatment with etoposide (approximately 1.5 to 1.6 kb) (arrow B) was detected in 2 peripheral blood samples of infants. Lanes 1 and 2, peripheral blood in infants; lane 3, cord blood; lane 4, peripheral blood in a child. C indicates control without stimulation of etoposide.

not successful in other samples because of the low level of PCR product. Because the DSB sites were identical in 2 samples each, 2 different nucleotide sequences of the DSB ends of the *MLL* gene were detected, as shown in Figure 3. Both DSBs were located on the telomeric side of intron 8 within the *MLL* BCR, and these locations can result in the fragment observed with Southern blot analysis. Although the topo-II consensus binding sequence detected with the high-stringency criteria [21] was not located on or near these DSB ends, 6/10 similarity on one DNA strand was observed within 40 bp of both DSB ends in intron 8 (Figure 3).

Table 1.
Incidence of Detectable Double Strand Breaks in the *MLL* Gene Caused by the Topoisomerase II Inhibitor Etoposide

	Concentration of Etoposide, μM			
Sample	0	1.0	10	50
Cord blood (n = 13)	0/4	1/3	1/3	1/3
Peripheral blood from infants (n = 11)	0/3	1/3	1/2	3/3
Peripheral blood from children (n = 14)	0/4	1/4	2/3	1/3

4. Discussion

Although an association between in utero *MLL* gene rearrangements and infant leukemia has been investigated, the precise mechanism remains controversial [7,8]. Results of a recent international epidemiologic study of infant leukemia confirmed that ingestion of several drugs, including herbal medicines and DNA-damaging drugs, or exposure to pesticides during pregnancy causes *MLL* gene fusion, possibly through an effect on topo-II [9]. Another case-control study showed that maternal dietary consumption of topo-II inhibitors was associated with infant leukemia [22]. The major concern has been, therefore, whether hematopoietic cells, like other cells, could be susceptible in the fetal period to *MLL* DNA breakage by topo-II inhibitors and whether the damage could be a cause of infant leukemia.

It has been suggested that topo-II inhibitors induce chromosomal breakage within the *MLL* gene and that *MLL* translocations result from mistakes in the DNA repair process [23]. Aplan et al [11] recently identified a site within the *MLL* BCR that was highly sensitive to DSBs induced by topo-II inhibitors (teniposide, etoposide, or doxorubicin). This site-specific cleavage, which mapped to the same region as a topo-II consensus site within exon 9 of the *MLL* gene, also was found in normal peripheral MNCs [11]. In the present study, we used etoposide in a Southern blot analysis to induce cleavage of the *MLL* gene in normal MNCs. The pattern and the size of fragment detected with the analysis were consistent with a DNA DSB occurring on the telomeric side of intron 8 and near exon 9 [11,18,20]. A faint but similar fragment was obtained from normal MNCs in previous studies [11,20]. A clearer and more persuasive fragment of DNA cleavage is the yield when leukemic or Epstein-Barr virus–transformed cell lines are used [11,20], because the instability of various genes may be high in these cells. Therefore, Southern blot analysis is not suitable for the detection of *MLL* gene cleavage in normal fresh samples. In experiments with the CEM cell line, the fragment of the *MLL* gene produced by treatment with etoposide was initially detected 8 hours after the treatment [11]. It is unlikely that these cells repaired their cleavage and thereafter proliferated to a detectable level within such a short time. It can be speculated that DNA cleavage can be simultaneously induced at similar sites of the *MLL* gene in each cell.

Lovett et al [24] demonstrated with in vitro assays that not only etoposide but also its metabolites enhance topo-II cleavage near the *MLL* BCR. The effect of other topo-II inhibitors, including flavonoids, catechins, caffeine, benzene derivatives, and herbal medicines, has been investigated [25]. Strick et al [12] reported that several dietary bioflavonoids can induce cleavage of the *MLL* gene in human progenitor cells and that these sites colocalize with cleavage sites induced by chemical topo-II inhibitors. These findings suggested that site-specific cleavage within the *MLL* BCR induced by topo-II inhibitors may be an early step leading to *MLL* gene rearrangements.

In the present study, we focused on in vitro breakage by etoposide on the telomeric side of intron 8 within the *MLL* BCR, and we identified the DSBs in normal blood MNCs. Although reproducible data on site-specific *MLL* cleavage

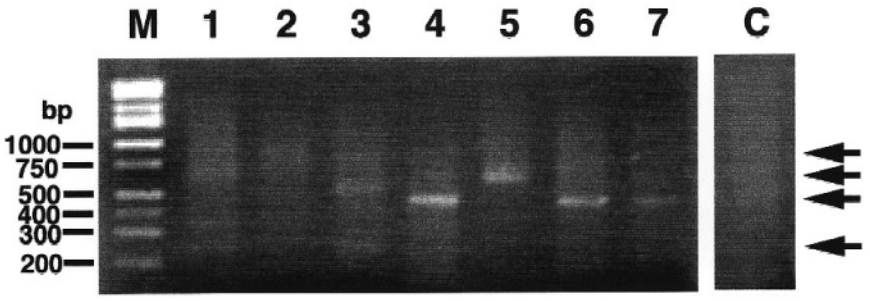

Figure 2. Ligation-mediated polymerase chain reaction (LM-PCR) of normal peripheral and cord blood samples treated with etoposide. To detect double strand breaks (DSBs) in DNA, LM-PCR was performed with BW linker and *MLL* gene–specific primers located in intron 8. Each PCR product was electrophoresed and visualized with ethidium bromide. Products of the same or different sizes showing DSBs of the *MLL* gene were found in several normal blood samples (arrows). Lane 1, peripheral blood of a child with 1 μM of etoposide; lane 2, cord blood with 50 μM of etoposide; lane 3, cord blood with 1 μM of etoposide; lane 4, peripheral blood of a child with 1 μM of etoposide; lane 5, peripheral blood of an infant with 10 μM of etoposide; lane 6, peripheral blood of a child with 1 μM of etoposide; lane 7, peripheral blood of an infant with 50 μM of etoposide. M indicates marker; C, control.

were not obtained in this study because only a small number of MNCs were available in each sample, it was clarified that *MLL* cleavage is induced by etoposide in both peripheral and cord blood MNCs. The possibility can be excluded that *MLL* cleavage was caused by artifacts such as DNA degradation due to ethidium bromide with ultraviolet light in a genomic DNA preparation or by loading for agarose gel electrophoresis, because control samples not treated with etoposide did not show any products in the LM-PCR.

The presence of several DNA motifs or structures in the *MLL* BCR may be an explanation for the DSBs induced by topo-II inhibitors: heptamer/nonamer-like sequences, scaffold attachment regions (SARs), topo-II consensus binding sites, and Alu repeats. The *MLL* BCR contains 3 Alu repeat elements: 1 in intron 6 and 2 in intron 8 [26]. Most t(4;11) translocation breakpoints are located in introns 6 and 8. However, Alu repeats have been responsible for the tandem duplication of the *MLL* gene usually observed in patients with acute myelogenous leukemia. This results from genomic fusion of introns 6 or 8 to intron 1 of the *MLL* gene [27]. Results of in vitro studies have shown that DNA cleavage induced by topo-II inhibitors also clustered in intron 6 or 8 [11,12,24]. Broeker et al [17] analyzed the distribution of breakpoints of the *MLL* gene in de novo acute leukemia and therapy-related leukemia. Most of the breakpoints in therapy-related leukemia mapped to the telomeric half of

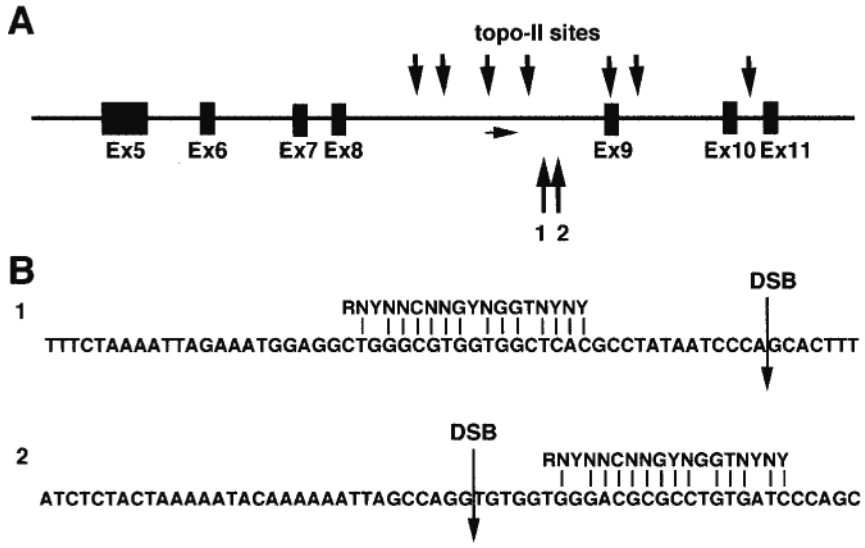

Figure 3. Double strand break (DSB) sites of the *MLL* gene in normal peripheral and cord blood samples identified by sequencing of polymerase chain reaction (PCR) products. PCR products were subcloned into pCRII. Plasmid DNA was extracted, and insert DNAs were sequenced with M13 forward and reverse primers. Sequencing analysis was successful in 4 samples; 2 different DSB sites from 2 samples each were located on the telomeric side of intron 8 within the *MLL* breakpoint cluster region, where the topoisomerase II (topo-II) consensus sites clustered (A). According to the criteria of the topo-II consensus site, only a 6/10 similarity on one DNA strand was observed near these cleavage sites (B). Horizontal arrow indicates the site of the *MLL*-specific primer for LM-PCR. Ex indicates exon; DSB, double strand break.

the *MLL* BCR that contains both the SARs and the topo-II consensus sites, whereas they did not map to the telomeric half in de novo acute leukemia [17]. According to the high-stringency criteria of topo-II consensus binding sites (9-10/10 similarity on one DNA strand), 4 breakpoints are located in intron 8, 1 in exon 9, 1 in intron 9, and 1 in intron 10, suggesting a significant role for topo-II consensus sites in therapy-related leukemia [17]. Strissel et al furthermore mapped an additional structure, a DNase hypersensitive site, near exon 9 within the *MLL* BCR [18]. They therefore proposed that topo-II is enriched in the *MLL* telomeric SARs and that it cleaves the DNase site with topo-II inhibitors [18]. Interestingly, most of the breakpoints in infant leukemia with *MLL* gene rearrangements also mapped to the telomeric half of the *MLL* BCR [19]. We identified 2 different cleavage sites on the telomeric side of intron 8 by means of in vitro treatment of cord and peripheral blood MNCs with etoposide. In therapy-related leukemia and infant leukemia, several cleavage sites have been identified, mainly in introns 6 and 8, but the exact location varied among the patients [6,28,29]. The *MLL* breakpoints usually cluster near the topo-II consensus sites rather than precisely at these sites. In the present study, only 6/10 similarity of one DNA strand was detected near the DSB sites of the *MLL* gene. The DNA cleavages, which occur at the topo-II sites, may be followed by a variable amount of exonucleolytic DNA degradation and subsequent relegation to a different chromosome and may produce a translocation [20].

We initially hypothesized that cord blood is more susceptible to *MLL* DNA cleavage by topo-II inhibitors than is peripheral blood, because the developing fetus is remarkably sensitive to topo-II inhibitors because of the high degree of cell turnover. In the present study, however, cleavage of the *MLL* gene in vitro was observed equally in cord and peripheral blood of infants and children. The fact that a low concentration of etoposide also induced *MLL* cleavage in hematopoietic cells can explain the susceptibility to DNA breaks during the fetal period. In addition, the DSBs of the *MLL* gene did not always occur in hematopoietic cells. The DNA cleavage in lymphocytes induced by topo-II inhibitors usually is reversible. Most of the *MLL* DNA cleavage is repaired correctly, but a reversible stage of the cleavage may lead to illegitimate chromosome translocations that result in leukemia [12,20].

Our previous study demonstrated that a high frequency of expression of in-frame fusion transcripts of the *AF4* gene, a notable partner gene for recombination with the *MLL* gene, was observed in most of the cord blood, whereas it was not observed in normal peripheral blood [30]. Interestingly, although infant leukemia and therapy-related leukemia have common breakpoints in *MLL*, they exhibit a striking difference in the partner genes [31]. Instability of the *AF4* gene during the fetal period may precede the *MLL* gene rearrangement in infant leukemia, resulting in a high frequency of *MLL/AF4*-positive infant leukemia.

In conclusion, in vitro cleavage in the *MLL* gene in cord and in peripheral blood can be equally induced with a low concentration of topo-II inhibitor. The DSB sites were detected on the telomeric side of intron 8 and near exon 9 within the *MLL* BCR, where the DNA cleavage was reported to occur in patients with therapy-related leukemia and in patients with infant leukemia. Cleavage in utero of the *MLL* gene induced by topo-II inhibitors and subsequent recombination with the unstable partner gene (*AF4*) may be an essential step in infant leukemia.

Acknowledgments

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