Etoposide induces MLL rearrangements and other chromosomal abnormalities in human embryonic stem cells

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MLL rearrangements are hallmark genetic abnormalities in infant leukemia known to arise in utero. They can be induced during human prenatal development upon exposure to etoposide. We also hypothesize that chronic exposure to etoposide might render cells more susceptible to other genomic insults. Here, for the first time, human embryonic stem cells (hESCs) were used as a model to test the effects of etoposide on human early embryonic development. We addressed whether: (i) low doses of etoposide promote MLL rearrangements in hESCs and hESC-derived hematopoietic cells; (ii) MLL rearrangements are sufficient to confer hESCs with a selective growth advantage and (iii) continuous exposure to low doses of etoposide induces hESCs to acquire other chromosomal abnormalities. In contrast to cord blood-derived CD34+ and hESC-derived hematopoietic cells, exposure of undifferentiated hESCs to a single low dose of etoposide induced a pronounced cell death. Etoposide induced MLL rearrangements in hESCs and their hematopoietic derivatives. After long-term culture, the proportion of hESCs harboring MLL rearrangements diminished and neither cell cycle variations nor genomic abnormalities were observed in the etoposide-treated hESCs, suggesting that MLL rearrangements are insufficient to confer hESCs with a selective proliferation/survival advantage. However, continuous exposure to etoposide induced MLL breaks and primed hESCs to acquire other major karyotypic abnormalities. These data show that chronic exposure of developmentally early stem cells to etoposide induces MLL rearrangements and make hESCs more prone to acquire other chromosomal abnormalities than postnatal CD34+ cells, linking embryonic genotoxic exposure to genomic instability.

Introduction

The MLL gene located in chromosome 11q23 fuses to generate chimeric genes with >50 partners in human leukemia (1–5). MLL rearrangements are particularly common in infant acute leukemias (6,7) and in secondary therapy-related acute leukemias (t-AL) associated with prior exposure to DNA topoisomerase II inhibitors (8). Elegant studies on identical twins with concordant MLL-AF4+ leukemia and retrospective analyses of the clonotypic MLL-rearranged sequence of leukemic cells from young patients in their neonatal blood spots revealed an in utero origin of the MLL rearrangements (9,10). MLL-rearranged leukemias are associated with poor prognosis and very brief latency for MLL-AF4+ infant B ALL. This raises the question of how this disease can evolve so quickly, particularly, if additional secondary mutations are required. MLL-rearranged leukemias commonly have activating FLT3 mutations (11,12) and ~50% of the cases have additional chromosomal abnormalities (7,13).

Epidemiological and genetic studies support the contention that the in utero origin of MLL rearrangements in infant leukemia may be the result of transplacental exposures during pregnancy, to substances that alter the function of DNA topoisomerase II (14,15). This parallels the origin of t-AL (8,15) and is supported by the finding that MLL rearrangements can be induced in vitro by etoposide, a DNA topoisomerase II inhibitor (16–20). Etoposide alters the function of the DNA topoisomerase II, which is highly expressed in developing fetuses (21). Etoposide is a widely used chemotherapy agent responsible for 5–15% of t-AL (2). Exposure of cells to topoisomerase II inhibitors increases the frequency of illegitimate recombination events (22), a physiological activity that may be related to both cytotoxicity and leukemogenicity of etoposide. Recent studies (23,24) suggest that high dietary intake of bioflavonoids, an abundant source of topoisomerase II inhibitors in the diet, could cause breaks in MLL and possibly in other partner genes, therefore playing an important role in the generation of the preleukemic clone in infancy and in the development of t-AL (25).

It has been shown that exposure to high doses of etoposide experimentally induces MLL breaks in mouse embryonic stem cells (mESCs) (16), fetal liver-derived CD34+ hematopoietic stem cells (HSCs) (19) and cord blood (CB)-derived CD34+ HSCs (17,18,20). However, the effects of etoposide earlier during human embryonic development remain to be determined. Human embryonic stem cells (hESCs) hold the promise to become a powerful tool for drug screening and toxicity but also to predict the onset of diseases known to begin during embryonic/fetal stages of development (26).

Here, for the first time, we have used hESCs as a model to test the effects of etoposide on human early embryonic development. We aimed at addressing whether: (i) very low doses of etoposide promote MLL rearrangements in hESCs and hESC-derived hematopoietic cells; (ii) MLL rearrangements are sufficient to confer hESCs with a selective proliferation/survival advantage and (iii) continuous exposure to very low doses of etoposide predisposes hESCs to acquire other chromosomal abnormalities. Briefly, a single low dose of etoposide induced a pronounced cell death and MLL gene fusions in undifferentiated hESCs and their hematopoietic derivatives. The MLL rearrangements were insufficient to confer the hESCs with a selective growth advantage. However, continuous exposure to etoposide promotes similar vulnerability to MLL rearrangements and primed hESCs, but not postnatal CB-derived CD34+ HSCs, for acquiring other major chromosomal abnormalities. Our findings suggest that continuous exposure to topoisomerase II inhibitors during human embryonic development induce MLL rearrangements and other chromosomal abnormalities in hESCs, linking embryonic genotoxic exposure to genomic instability.

Material and methods

hESC culture

hESCs were maintained undifferentiated in a feeder-free culture as described previously (27–29). Briefly, hESCs were cultured in Matrigel (BD Biosciences, Bedford, MA)-coated T25 flasks in human foreskin fibroblast-conditioned medium supplemented with 8 ng/ml basic fibroblast growth factor (Invitrogen, Burlington, Ontario, Canada). Human foreskin fibroblast-conditioned medium was changed daily, and the cells were split weekly by dissociation with 200 U/ml of collagenase IV (Invitrogen). Distinct hESC cultures (~70% confluent) were exposed to a single dose of 0.2 or 0.5 µM etoposide (Sigma–Aldrich, St Louis, MO) or dimethyl sulfoxide (DMSO) as a vehicle control for 16 h. Then, etoposide was washed away and the cells were allowed to recover in human foreskin fibroblast-conditioned medium.
(Figure 1A). For continuous etoposide exposure, hESCs were exposed to an initial dose of 0.2 μM followed by pulses of 0.02 μM of etoposide every 3 days for 40 days. Approval from the Spanish National Embryo Ethical Committee was obtained to work with hESCs.

**CB-derived CD34⁺ isolation and culture**

Different fresh CB samples from healthy newborns were obtained from local hospitals upon approval by our local Ethics and Biohazard Board Committee. CB samples were pooled to reduce variability between individual freshly

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**Fig. 1.** Effects of a single low dose of etoposide in hESC viability and MLL gene rearrangements. (A) Experimental design for etoposide treatment of hESCs. Stable hESCs were exposed to either 0.2 or 0.5 μM of etoposide for 16 h. Etoposide-treated hESCs were allowed to expand. Then, cellular viability, MLL breaks, cell cycle and genomic stability were assessed. (B and C) Cellular viability and morphology, as determined under the stereoscope and light microscopy, revealed 80 and 95% cellular death upon 0.2 and 0.5 μM etoposide treatment, respectively. (D) MLL breaks were identified and quantified (E) by using a MLL split-apart probe by interphase fluorescence in situ hybridization (iFISH). DMSO-treated cells displayed two yellow signals corresponding to germline alleles, whereas etoposide-treated cells display one yellow signal (germline) and one green signal and one red signal representing typical MLL breaks. White arrows point to MLL breaks in etoposide-treated hESCs.
isolated CB units. Mononuclear cells were isolated using Ficoll–Hypaque (GE Healthcare, Stockholm, Sweden). After lysing the red blood cells (Lysis solution, StemCell Technologies, Vancouver, Canada), CD34+ cells were purified by magnetic bead separation using the human CD34 MicroBead kit (Miltenyi, Madrid, Spain) and the AutoMACS Pro separator (Miltenyi) as per manufacturer’s instructions. In four experiments, cytokine-stimulated CD34+–enriched fraction was plated (0.2–0.5 × 10^7/cm²) and exposed to a single dose of 0.2 or 0.5 μM etoposide or DMSO as vehicle for 16 h. Then, CD34+–treated cells were plated in methylcellulose supplemented with stem cell factor (50 ng/ml), granulocyte-macrophage colony-stimulating factor (10 ng/ml), interleukin (IL)-3 (10 ng/ml) and erythropoietin (3 U/ml) (Methocult GF H4434 StemCell Technologies). After 14 days in primary colony forming unit assay, the cells were harvested and released in secondary colony forming unit assay for another 14 days and assessed for MLL rearrangements. For continuous etoposide exposure, in two independent experiments, CB–derived CD34+ cells (0.2–0.5 × 10^7/cm²) were pooled and cultured in serum-free medium StemSpan (StemCell Technologies) supplemented with stem cell factor (100 ng/ml), granulocyte-colony stimulating factor (50 ng/ml), FMS-like tyrosine kinase 3 (FLT-3) ligand (100 ng/ml), thrombopoietin (20 ng/ml) and IL-6 (20 ng/ml) (Peprotech, London, UK). Etoposide at 0.04 μM was added to the media every 3 days up to 30 days, after an initial hit with 0.2 μM of etoposide.

**Interphase fluorescence in situ hybridization studies**

Interphase fluorescence in situ hybridization was performed using a LSI MLL Dual Color, break-apart rearrangement probe (Izasa, Barcelona, Spain). The probe was pipetted on top of the cells and denatured at 73°C for 3 min. The slides were then incubated at 37°C for 4 h in a moist chamber; afterward, the covers were removed and the slides were washed at 73°C for 5 min in 0.4× SSC/0.1% NP-40 followed by 3× SSC/0.1% NP-40 at room temperature for 1 min. The slides were then air-dried in the darkness, covered with anti-fade solution and sealed with a cover glass. For each sample, at least 500 nuclei were analyzed. The slides were analyzed in a fluorescence microscope equipped with appropriate filters using the ISIS-software (Metasystems, Altlussheim, Germany).

**Conventional karyotyping**

Cells were cultured in medium supplemented with 0.1 mg/ml colcemid for up to 3–4 h. The cells were then washed in verse solution and subsequently trypsinized and spun down. The pellet was resuspended carefully in a KCl hypotonic solution, rinsed to remove the cytoplast and then fixed in methanol/acetic acid 3:1. Finally, the pellet was resuspended in a final volume of 1 ml of fixative and cells dropped onto glass slides. Chromosomes were visualized by using a modified Wright's staining. At least 20 metaphases were analyzed for each cell line (30–32) using a conventional microscope and the IKAROS-software (Metasystems).

**Spectral karyotyping analysis**

For spectral karyotyping (SKY) analysis, metaphases were prepared as described above. Slides were hybridized using the SKY method according to the manufacturer’s protocol (Applied Spectral Imaging, Migdal Ha’Emek, Israel). Images were acquired with an SD300 Spectra Cube (Applied Spectral Imaging) mounted on a Zeiss Axioplan microscope using a custom-designed optical filter (SKY-1; Chroma Technology, Brattleboro, VT). Breakpoints on the SKY–painted chromosomes were determined by comparison of corresponding 4′,6-diamidino-2-phenylindole banding and by comparison with G-banding karyotype (30–32).

**FLT3 gene mutation analysis**

The three most common activating mutations of the FLT3 tyrosine kinase receptor [D835Y, I836del and internal tandem duplication (ITD)] were assessed by polymerase chain reaction (PCR) as described previously (33). The primers used were described previously (33). For FLT3-ITD, genomic DNA was amplified to produce a fragment of 238 bp from germline alleles and a longer (260 bp) fragment from mutated FLT3-ITD alleles. PCR conditions were as follows: 35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C. FLT3 point mutations at codons 835 or 836 were detected by PCR amplification followed by EcoRV digestion to produce a unique fragment of 199 bp. The PCR conditions used were as above, but the annealing temperature was 60°C.

**Cell cycle analysis**

hESCs were harvested and pelleted by centrifugation. Cell pellets were washed with phosphate-buffered saline, fixed in 70% ice-cold ethanol and stored at −20°C for up to 2 weeks at −20°C. Cells were washed with phosphate-buffered saline followed by incubation in 50 μg/ml propidium iodide and 100 μg/ml RNase A (Sigma) for 30 min. Stained nuclei were analyzed on a fluorescence activated cell sorting Canto-II using the fluorescence activated cell sorting Diva software (Becton Dickinson, San Jose, CA).

**Long distance inverse–PCR studies**

Long distance inverse (LDI)–PCR was used to confirm the presence of MLL gene fusions within the MLL break cluster region upon etoposide treatment. Genomic DNA was digested with either BamHI or XbaI (Figure 2). LDI–PCR reactions were performed as described previously (17,34). Germline MLL PCR products were eliminated by digesting the circularized DNA with NsiI (BamHI) or PvuII (XbaI), respectively. Primers used were as follows: A-5′-GACATCTCCCCTTCTACCTCTTTCC-3′; D-5′-ATCTCGAATAAATGCGACCTTTGTGTTGTG-3′; MF1-5′-CTCTAAACGTGCCAAGGCTTCT-3′; MF2-5′-ATAATGCTGTCAGTCTGCAGTCTCAAT-3′; MR1-5′-CCCCAGTGGATTTCTTCTTA-3′ and MR2-5′-GATGAGATGTGCTCTTAPAAATGACAAACTACTGTCTCC-3′ (Figure 2). Although this procedure was useful for eliminating amplification from the germline MLL allele, it does not identify rearrangements that contain an Nsil or PvuI sites from the partner gene within the circularized DNA template. Individual LDI–PCR products (n = 28) were excised and purified from agarose gels, cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced with M13 forward and reverse primers. Sequences were analyzed using BLAT (UCSC Genome Bioinformatics, Santa Cruz, CA) at http://genome.ucsc.edu.

**Etoposide treatment of hESC–derived hematopoietic progeny**

Undifferentiated hESCs at confluence were treated with collagenase IV and scraped off the Matrigel attachments. They were then transferred to low–attachment plates to allow embryoid body formation by overnight incubation in differentiation medium consisting of knock-out Dulbecco’s modified Eagle’s medium supplemented with 20% non–heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1 mM l-glutathione and 0.1 mM β-mercaptoethanol. The medium was changed the next day (day 1) with the same differentiation medium supplemented with hematopoietic cytokines [300 ng/ml stem cell factor, 300 ng/ml FLT-3 ligand, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml granulocyte-colony stimulating factor and 25 ng/ml bone morphogenetic protein-4] (29).

Single cells dissociated from 15 day hEBs were plated in fibronectin-coated plates (Becton Dickinson) and expanded up to day +22 in liquid culture (StemSpan media, StemCell Technologies) supplemented with the above cytokines. hESC–derived hematopoietic progeny was treated for 16 h with either 0.5 μM etoposide or DMSO on day +16 or day +19 of hematopoietic differentiation. Washing away the etoposide, cells were allowed to recover in StemSpan medium containing cytokines. Four days later, etoposide- and DMSO-treated hESC–derived hematopoietic cells were harvested and the immunophenotype and MLL breaks were assessed. The proportion of CD45+ hematopoietic cells on day +15 and day +22 of hESC hematopoietic development was 45% and 86%, respectively.

**Results**

A single low dose of etoposide induces MLL rearrangements and pronounced cell death in hESCs

We first addressed whether very low doses of etoposide induce MLL rearrangements on hESCs that represent the earliest stage of human embryonic development available for study. To determine the effect of etoposide, hESCs were exposed for 16 h to a single low dose of etoposide (0.2 or 0.5 μM). After the 16-h treatment, etoposide was washed away and the cells were allowed to recover (Figure 1A). Five days after treatment, a pronounced cell death was observed in etoposide-treated hESCs (Figure 1B). As compared with DMSO-treated hESCs, cell death was 80% and >95% in 0.2 and 0.5 μM etoposide-treated hESC cultures, respectively (Figure 1C). hESCs that had survived the etoposide treatment and were able to expand after exposure in the recovery period displayed identical undifferentiated morphology to DMSO-treated hESCs (Figure 1B).

**MLL gene was found rearranged by interphase fluorescence in situ hybridization in 1.5 and 2.4% of the cells exposed for 0.2 and 0.5 μM of etoposide, respectively (Figure 1D and E and Table I). Using a break-apart FISH probe, a split signal with one green and one red dot represents a MLL break, whereas the germline MLL allele appears as a single yellow signal (Figure 1D and E).** LDI–PCR analysis confirmed that etoposide treatment induced MLL gene rearrangements at intron 11 within the MLL break cluster region (Figure 2A and B). Cloning and sequencing of 28 LDI–PCR products revealed that the MLL gene rearranged with random intergenic regions such as 4q31.3 and 7q31.1 (Figure 2C). The fusion probably occurred through non-homologous end joining due to the lack of homology at the junction (supplementary Figure 1 is available at Carcinogenesis Online). As both identified MLL rearrangements occurred within genomic regions...
Etoposide induces MLL rearrangements and other chromosomal abnormalities in hESCs

devoid of human genes, it is very unlikely that those MLL fusions give rise to stable messenger RNA transcripts that would be difficult to detect by conventional expression analyses. However, this in vitro data support the proof-of-principle that a single very low dose of etoposide is highly toxic to hESCs and induces MLL rearrangements in the surviving hESCs.

Table I. Etoposide-induced MLL Breaks in hESCs and CB-derived CD34\(^+\) cells after short-term and long-term recovery periods

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<tr>
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<th>Short term (%)</th>
<th>Long term (%)</th>
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<td><strong>hESCs</strong></td>
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<td>DMSO</td>
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<td>Etoposide 0.2 (\mu)M</td>
<td>0.3–3</td>
<td>0.2–1.2</td>
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<td>Etoposide 0.5 (\mu)M</td>
<td>0.7–3.2</td>
<td>0.5–1.4</td>
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<td>CB-derived CD34(^+) cells</td>
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<tr>
<td>DMSO</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Etoposide 0.2 (\mu)M</td>
<td>0.1–1.8</td>
<td>0.1–1.2</td>
</tr>
<tr>
<td>Etoposide 0.5 (\mu)M</td>
<td>0.6–2.7</td>
<td>0.5–2</td>
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Five hundred nuclei were analyzed in each sample.

Etoposide-induced MLL rearrangements are not sufficient to confer hESCs with a selective proliferation/survival advantage

We next investigated whether etoposide-induced MLL rearrangements are sufficient to confer hESCs with a selective proliferation/survival advantage. Four to five weeks after etoposide exposure, we assessed the overall genomic stability of surviving hESCs. We first analyzed the cell cycle distribution by flow cytometry. No differences between etoposide- and DMSO-treated hESCs were observed: 36.9 and 35.6\% of the cells were in G\(_2\)/M phase and 63.1 and 64.4\% of the cells were in S/G\(_1\) phase, respectively (Figure 3A).
Using molecular cytogenetics, no discernible numerical or structural chromosomal abnormalities were observed by conventional G-banding (Figure 3B) and SKY (Figure 3C) in the surviving hESCs. MLL-rearranged leukemias commonly present FLT3-activating mutations that seem to cooperate with MLL in leukemogenesis (11,12,35). Activating FLT-3 mutations were analyzed in both DMSO- and etoposide-treated hESCs. As shown in Figure 3D, etoposide exposure did not induce FLT-3-activating mutations even 5–6 weeks after exposure.

To further address whether etoposide-treated hESC subset harboring a rearranged MLL gene acquired proliferative and/or survival advantage, the percentage of MLL-rearranged cells within the bulk

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Fig. 3. Overall genomic stability of hESCs exposed to a single low dose of etoposide. (A) Analysis of the cell cycle distribution of etoposide-treated versus DMSO-treated hESCs was assessed by propidium iodide staining. No differences were found in the cell cycle distribution in etoposide-treated hESCs. (B) Conventional G-banding revealed euploid DMSO- and etoposide-treated hESCs. (C) Using SKY, neither numerical nor structural chromosomal abnormalities were observed in etoposide-treated hESCs. (D) Etoposide-treated hESCs lack both FLT3 point mutations (left panel) and FLT3-ITD mutations (right panel).
hESC culture was determined short term (4 weeks) and long term (12 weeks) after exposure. As indicated in Table I, the proportion of hESCs carrying a rearranged \textit{MLL} allele diminished from week 4 to week 12 after etoposide treatment. This loss of \textit{MLL}-rearranged hESC clones over time along with the overall genomic stability in etoposide-treated cultures suggests that although a single very low dose of etoposide is capable of inducing \textit{MLL} rearrangements in a subset of hESCs, it does not confer either proliferative or survival advantage and therefore it is not sufficient to promote \textit{in vitro} cell transformation of hESCs under our experimental conditions.

\textbf{Continuous exposure of hESCs to very low doses of etoposide induces other major chromosomal abnormalities}

According to the proposed ‘two-hit’ model for the natural course of childhood leukemia (35), the natural history of pediatric leukemia usually involves two events. The initiating event is frequently a chromosomal translocation that occurs prenatally, followed by a secondary mutation that promotes overt disease. The very short latency observed in infant leukemias with \textit{MLL} rearrangements suggests that the second hit might occur prenatally as a consequence of continuous exposure to the same genotoxic agent that the one which induced the \textit{MLL} fusion (7). We thus analyzed whether chronic exposure to extremely low doses of etoposide might promote other genomic abnormalities in hESCs. hESCs received an initial dose of 0.2 \mu M of etoposide followed by a 0.02 \mu M dose every 4 days for a period of 42 days. As shown in Figure 4A, the percentage of \textit{MLL} gene fusions was similar to those observed after a single hit (2.4%; range: 1.5–2.8%). Interestingly, G-banding (Figure 4B) and SKY (Figure 4C) analyses revealed that chronic exposure to very low doses of etoposide induced other major chromosomal abnormalities, including trisomies and translocations. Representative complex karyotypes were 47 XX t(1;6) (q21;q26); i(1)(q10) or 46 XX t(4;11)(p15;q12); t(1;6) (q21;q26) (Figure 4B and C). This data represents the first \textit{in vitro} experimental evidence supporting the proof-of-principle that chronic exposure of human prenatal stem cells to etoposide contribute to an increased incidence of chromosomal damage.

\textbf{CB-derived CD34\textsuperscript{+} cells display differential susceptibility to etoposide exposure compared with undifferentiated hESCs or hESC-derived hematopoietic derivatives}

In order to test the susceptibility of postnatal CD34\textsuperscript{+} HSC cells to single or chronic exposure to etoposide, pooled CD34\textsuperscript{+} HSCs were

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\caption{Chromosomal alterations in hESCs in response to continuous exposure to etoposide. (A) The incidence of \textit{MLL} rearrangements detected 8 weeks after continuous treatment with etoposide was 2.4\% (left panel). Representative example of \textit{MLL} breaks after continuous exposure of hESCs to etoposide (right panel). (B) Representative random chromosomal abnormalities detected by conventional G-banding karyotype: 47 XX, t(1;6)(q21;q26), i(1)(q10) (top panel) or 46 XX, t(1;6)(q21;q26), t(4;11)(p15;q12) (bottom panel). (C) Chromosomal alterations were confirmed by SKY.}
\end{figure}
exposed for 16 h to a single low dose of 0.2 or 0.5 μM of etoposide, respectively (Figure 5A). Treated cells were expanded in methylcellulose or liquid culture containing hematopoietic cytokines. In contrast to the pronounced cytotoxic effect that the etoposide exerted on hESCs (Figure 1B), it barely induced cell death in CB-derived CD34^+ cells. Cell death was always <20%, regardless the dose of etoposide (Figure 5B). Regarding the incidence of MLL rearrangements, the MLL gene was found rearranged in a slightly lower percentage of CB-derived CD34^+ cells as compared with hESCs: 1.2% (range: 0.1–1.8%) and 1.8% (range: 0.6–2.7%) of the CB-derived CD34^+ cells treated with 0.2 and 0.5 μM of etoposide, respectively (Figure 5C and Table I). Similar to hESC cultures, the percentage of MLL-rearranged CD34^+ cells slightly decreased after long term in culture (Table I).

We next addressed whether continuous exposure of CB-derived CD34^+ cells to low doses of etoposide facilitates further chromosomal abnormalities as observed in hESCs. CD34^+ cells received an initial dose of 0.2 μM of etoposide followed by a 0.04 μM dose every 3 days. Similar to CD34^+ cells receiving a single hit of etoposide, 2% of the CD34^+ cells exposed to a chronic exposure of etoposide displayed MLL breaks (Figure 5D). However, G-banding (Figure 5E) and SKY analyses (Figure 5F) revealed that continuous exposure to etoposide failed to induce further numerical or structural chromosomal abnormalities as observed with hESCs.

It has been suggested that early prenatal HSCs may be the target for MLL fusions. Therefore, using conditions previously optimized to promote hematopoietic differentiation from hESCs (29,31), we assessed to what extend etoposide induces MLL breaks at two different developmental stages during human embryonic hematopoietic development. As shown in Figure 6, early (day +15) hESC-derived hematopoietic derivatives seem to be slightly more susceptible to etoposide-induced MLL breaks than late (day +22) fully differentiated hESC-derived hematopoietic derivatives (2.9 versus 1.6%, respectively). Taken together, these results suggest that postnatal CD34^+ and late hESC-derived hematopoietic cells are less vulnerable to etoposide-induced MLL rearrangements than undifferentiated hESCs or earlier hESCs-derived hematopoietic cells.

Discussion

A growing body of evidence supports the contention that the in utero origin of MLL rearrangements in infant leukemia may be the result of transplacental exposures during pregnancy, perhaps to substances that alter the function of DNA topoisomerase II such as etoposide (14,15,36,37). Recent studies (23,24) suggest that high dietary intake of bioflavonoids, an abundant source of topoisomerase II inhibitors, could cause breaks in MLL, therefore playing an important role in the...
generation of the preleukemic clone in infancy as well as in the development of t-AL (25). Exposure to high doses of etoposide induces MLL breaks in mESCs (16) and human postnatal CD34+ HSCs (17–20). However, the potential effects of etoposide earlier during human embryonic development remain to be assessed. In contrast to neonatal-CD34+ HSCs, exposure to a single low dose of etoposide induced a pronounced cell death in hESCs. As expected, this tremendous cell death of hESCs was observed 4–5 days after etoposide treatment due to the use of very low doses of etoposide and its mechanism of action. Etoposide exerts its cytotoxic effects by inhibiting the enzyme DNA topoisomerase. The absence of DNA topoisomerase has a cumulative negative effect on DNA repair, leading to many unrepaired breaks in cellular DNA and ultimately cell death. The striking vulnerability of hESCs to etoposide-induced cell death is in line with previous studies confirming the crucial role of both DNA topoisomerase II alpha and beta in human developing tissues (21). This high susceptibility to cell death of hESCs exposed to DNA topoisomerase II inhibitors may reveal clues to further understand the relationship between in utero exposure to genotoxic compounds and the risk of deleterious chromosomal abnormalities and lethal birth defects, commonly incompatible with tissue/embryo normal development, leading in many occasions to embryo loss and abortions (38).

A single low dose of etoposide induced MLL breaks in ~2–3% of the hESCs. These data are similar to that reported in fetal and CB-derived CD34+ HSC (17–20). Interestingly, however, hESCs are much more susceptible than mESCs to etoposide-induced MLL breaks (16). Etoposide concentrations are as high as 100 µM barely induced MLL rearrangements in just one of 62,500 mESCs, whereas relatively low concentrations (0.2–0.5 µM) induced MLL gene fusions in an average of three of 100 hESCs (a 1800-fold increase) (16). It is worth mentioning that physiological doses of etoposide were used because the etoposide concentration in the plasma of cancer patients treated with this drug ranges between 1–2 µM (39), a concentration far below from the 100 µM employed in previous studies. The LDI–PCR used by Blanco et al. (16) is more sensitive than the interphase fluorescence in situ hybridization method employed here to detect MLL fusions, further confirming the higher susceptibility of hESCs as compared with mESCs to etoposide-induced MLL breaks. Furthermore, hESCs and early hESC-derived hematopoietic derivatives seem to be more susceptible to etoposide-induced MLL breaks than later fully differentiated hESC-derived hematopoietic cells.

The demonstration that hESCs and their early hematopoietic derivatives are susceptible to etoposide-induced MLL rearrangements indicates that embryonic exposure to DNA topoisomerase II inhibitors might expand our understanding about the cellular origin for MLL rearrangements that are known to arise in utero during embryonic/fetal development. These data also pinpoint that, at least in drug screening and toxicity studies, human cells offer an alternative to animal cells that may be more accurate and could help ferret out safety issues that animal models would fail to identify.

We next wondered whether the MLL rearrangements confer hESCs with a selective proliferation/survival advantage. After long-term culture, the proportion of hESCs harboring MLL breaks diminished and

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**Fig. 6.** Effects of etoposide on hESC-derived hematopoietic progeny MLL gene rearrangements. The graph compares the percentage of cells harboring MLL breaks upon etoposide treatment in undifferentiated hESCs, postnatal CB-CD34+ HSCs and hESC-derived hematopoietic cells at two developmental stages: day 15 and day 22 of the hematopoietic differentiation. Below, the immunophenotype (CD45+) of each cell subset is shown by flow cytometry.
neither cell cycle variations nor karyotypic changes or FLT3 mutations were observed in the etoposide-treated hESC cultures, suggesting that the MLL rearrangements are insufficient to confer hESCs with a selective proliferation/survival advantage. The proportion of hESCs carrying a rearranged MLL allele diminished from week 4 to week 12 after etoposide treatment. This slight loss in the proportion of MLL-rearranged hESC clones suggests that during the hESC culture homeostasis there is a balance between DNA repair and cell death. hESCs harboring broken but not fused MLL alleles may be unable to repair these breaks and would probably undergo cell death. It is also plausible that DNA damage would have already been repaired through non-homologous end joining (supplementary Figure 1 is available at Carcinogenesis Online). In this scenario, the hESC clones harboring a MLL allele would diminish because the MLL breaks do not allow the cells to proliferate at the same rate as the non-rearranged MLL cells.

Although continuous exposure to etoposide promotes similar vulnerability to MLL rearrangements in embryonic versus neonatal stem cells, hESCs but not CB-derived CD34+ HSCs acquired other numerical and structural chromosomal abnormalities. In contrast to CD34+ HSCs, hESCs showed an enhanced susceptibility to both etoposide-mediated cell death and an increased incidence of chromosomal instability upon chronic exposure. The lack of cell transformation in hESCs harboring MLL rearrangements along with other genomic aberrations suggest 2-fold: (i) the functional impact of the MLL fusion and resulting clonal expansion can occur downstream the cellular origin of the translocation and (ii) a specific MLL rearrangement (distinct to the MLL fusions we randomly induced in our experiments) with robust leukemogenic potential is required for transformation. Taken together, our data suggest that continuous exposure to topoisomerase II inhibitors during human embryonic development not only induce MLL rearrangements but also make hESCs more susceptible to an increased incidence of chromosomal insults, further linking embryonic chronic genotoxic exposure to genomic instability.

hESCs may become a powerful tool for drug screening and toxicity of potentially harmful drugs but also to predict the onset of diseases known to begin prenatally. This data should encourage the field to explore the potential of hESCs in drug discovery and in studies aimed at predicting the onset of diseases to unravel fundamental mechanisms underlying the etiology and pathogenesis of conditions known to arise in utero.

Supplementary material
Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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References


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