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Environmental and chemotherapeutic agents induce breakage at genes involved in leukemia-causing gene rearrangements in human hematopoietic stem/progenitor cells

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ABSTRACT

Hematopoietic stem and progenitor cells (HSPCs) give rise to all of the cells that make up the hematopoietic system in the human body, making their stability and resilience especially important. Damage to these cells can severely impact cell development and has the potential to cause diseases, such as leukemia. Leukemia-causing chromosomal rearrangements have largely been studied in the context of radiation exposure and are formed by a multi-step process, including an initial DNA breakage and fusion of the free DNA ends. However, the mechanism for DNA breakage in patients without previous radiation exposure is unclear. Here, we investigate the role of non-cytotoxic levels of environmental factors, benzene, and diethylnitrosamine (DEN), and chemotherapeutic agents, etoposide, and doxorubicin, in generating DNA breakage at the patient breakpoint hotspots of the *MLL* and *CBFB* genes in human HSPCs. These conditions represent exposure to chemicals encountered daily or residual doses from chemotherapeutic drugs. Exposure of HSPCs to non-cytotoxic levels of environmental chemicals or chemotherapeutic agents causes DNA breakage at preferential sites in the human genome, including the leukemia-related genes *MLL* and *CBFB*. Though benzene, etoposide, and doxorubicin have previously been linked to leukemia formation, this is the first study to demonstrate a role for DEN in the generation of DNA breakage at leukemia-specific sites. These chemical-induced DNA breakpoints coincide with sites of predicted topoisomerase II cleavage. The distribution of breakpoints by exposure to non-cytotoxic levels of chemicals showed a similar pattern to fusion breakpoints in leukemia patients. Our findings demonstrate that HSPCs exposed to non-cytotoxic levels of environmental chemicals and chemotherapeutic agents are prone to topoisomerase II-mediated DNA damage at the leukemia-associated genes *MLL* and *CBFB*. These data suggest a role for long-term environmental chemical or residual chemotherapeutic drug exposure in generation of DNA breakage at sites with a propensity to form leukemia-causing gene rearrangements.

1. Introduction

Leukemia is one of the leading causes of male and female cancer deaths in the United States. Most leukemias are thought to originate in hematopoietic stem or progenitor cells and are typically associated with a variety of genetic alterations, including gene rearrangements or copy number variations [1]. The formation of gene rearrangements that cause leukemia is a multi-step process, including the initial double-stranded DNA breakage and subsequent joining together of the gene pairs. Ionizing radiation has been shown to cause rearrangement-generating DNA breakage, but in patients without previous exposure to radiation, the mechanism is unknown. However, exposure to a number of environmental chemicals and chemotherapeutic agents has been linked to development of leukemia, making it one of the most common types of cancer induced by a variety of cancer-causing agents [1]. Interestingly, many of these same chemicals, including benzene, induce the expression of chromosomal fragile sites, suggesting a potential mechanism for generation of DNA breakage that leads

Abbreviations: HSPC, hematopoietic stem and progenitor cells; APH, aphidicolin; PBS, phosphate-buffered saline; DEN, diethylnitrosamine; BCR, breakpoint cluster region; LM-PCR, ligation-mediated PCR.

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to gene rearrangement [2]. While the two chemotherapeutic drugs etoposide and doxorubicin have not previously been linked directly to fragile site breakage, inhibition of their therapeutic target, DNA topoisomerase II, has been suggested to play a role in fragile site instability [3].

Chromosomal fragile sites are regions of the genome susceptible to breakage under conditions of replication stress. Fragile site breakage is typically induced in the laboratory by treatment of cells with low doses of the DNA polymerase inhibitor, aphidicolin (APH). Low-dose APH exposure leads to expression of over 230 fragile sites in human cells [4]. In addition to APH, fragile sites are prone to break following exposure to many chemicals, including environmental and chemotherapeutic agents (reviewed in [5]). Therefore, understanding both the mechanism for, and consequences of, fragile site breakage following exposure to such chemicals will be important in guiding treatment decisions and preventing exposures that may cause human disease.

Fragile site breakage is associated with a number of human diseases, including cancer. Many gene rearrangements and copy number variations in cancer occur at genes located within fragile sites [6,7]. The most frequently expressed fragile site, FRA3B, is located within the tumor suppressor *FHIT*, and exposure of cells to APH induces submicroscopic deletions within *FHIT* that are consistent with those seen in numerous cancers, including esophageal, breast, and small-cell and non-small cell lung carcinomas [8]. Furthermore, exposure of human thyroid cells to fragile site-inducing conditions leads to formation of the *RET/PTC* rearrangement that causes papillary thyroid carcinoma [9]. In addition to the *RET/PTC* rearrangement in papillary thyroid carcinoma, over half of cancer-causing gene rearrangements contain at least one gene located within a fragile site [10]. A large number of these rearrangements are associated with various leukemias, such as acute myeloid leukemia and acute lymphoblastoid leukemia, suggesting a role for fragile site breakage in leukemogenesis. Two leukemia-associated genes located within fragile sites are *MLL*, located within FRA11G, and *CBFB*, located within FRA16C. DNA breakage within *MLL* can lead to rearrangement with over 120 partner genes, resulting in acute leukemias with typically poor prognosis [11]. *MLL* rearrangements are found in approximately 10% of leukemias [12] and 25% of therapy-related AML [13]. Mapping of patient breakpoints has identified a breakpoint cluster region (BCR) spanning exons 8–12, with *de novo* breakpoints within a 3-kb region between exons 9 and 11, and therapy-related leukemia breakpoints clustering at the intron 11/exon 12 junction [11,14]. DNA breakage within *CBFB* can lead to the inv(16) and related t(16;16)(p13;q22) rearrangement found in approximately 10% of all *de novo* acute myeloid leukemias. All *CBFB* breakpoints involved in these rearrangements have been mapped to intron 5, and all are associated with the M4Eo subtype [15,16]. Many studies have demonstrated that rearrangement formation within hematopoietic stem and progenitor cells (HSPCs) can promote the formation of leukemia [17–20]; however, the role of fragile site breakage in the generation of these rearrangements has yet to be investigated.

HSPCs are the cells from which all cells of the hematopoietic system are derived [1]. These cells are capable of self-renewal and differentiation into mature blood cells found throughout the body. While HSPCs are typically found in the bone marrow, they are able to move throughout the peripheral blood to a number of tissues at low levels, and also are released in larger numbers in response to cytokines, growth factors, and under conditions of stress or myelosuppression [21–23]. The mobility of these cells throughout the body may leave them susceptible to exposure to DNA damaging agents, such as environmental chemicals or chemotherapeutic agents, which have the potential to lead to leukemogenesis.

The location of leukemia-associated genes within common fragile sites and the role of environmental chemicals and chemotherapeutic agents in fragile site instability, leukemogenesis, and topoisomerase interaction prompted us to investigate whether these compounds cause DNA breakage at leukemia-associated fragile sites. The sensitivity of the BCRs of *MLL* and *CBFB* to the environmental chemicals, benzene, and diethylnitrosamine (DEN), or the chemotherapeutic agents, etoposide, and doxorubicin, in human $CD34^+$ HSPCs was determined by ligation-mediated PCR (LM-PCR). The nucleotide positions of chemical-induced breakpoints were mapped to sites of topoisomerase II cleavage, using DNA secondary structure predictions and sequence analysis. The distribution of chemical-induced breakpoints in our studies was compared to that of fusion breakpoints found in leukemia patients. The results suggest a role for topoisomerase II in breakage at leukemia-associated fragile sites in human $CD34^+$ HSPCs in response to chemical agents linked to leukemogenesis, such as benzene, etoposide and doxorubicin, and also DEN, which has not been previously connected to leukemogenesis.

2. Materials and methods

2.1. Cells and culture conditions

Experiments were performed using primary human $CD34^+$ bone marrow stem and progenitor cells (ALLCELLS), grown in Hematopoietic Growth Medium (Lonza) supplemented with StemSpan CC100 (StemCell Technologies) containing IL-3, IL-6, Stem Cell Factor and FLT-3 ligand.

2.2. Cell treatments and analyses

To analyze cell viability, human $CD34^+$ cells (1×10^5) were plated in 12-well plates and treated immediately with chemical concentrations as indicated in the text for 24 h. Cells were collected, washed with phosphate-buffered saline (PBS; Invitrogen), and re-suspended in PBS containing 2 μg/mL propidium iodide. Cell viability was determined using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences). Various concentrations of benzene (0.5–1 mg/mL), diethylnitrosamine (DEN, 1.5–3.0 mg/mL), etoposide (150 nM–10 μM), and doxorubicin (5–700 nM) were examined to determine the optimal dosage for the treatment of $CD34^+$ cells.

To analyze active apoptosis, $CD34^+$ cells (1×10^5) were plated and treated as above for 24 h. Cells were then washed with PBS and re-suspended in 1X annexin V binding buffer. Annexin V (BD Biosciences) was then added to each sample and incubated in the dark for 15 min. Early apoptotic cells were quantified using a FACSCalibur flow cytometer.

To analyze cell growth, viable $CD34^+$ cells (0.65×10^5) were determined by trypan blue exclusion using a hemocytometer, plated, and treated in 12-well plates. Cells were harvested after 24 h, washed with PBS, and re-plated with fresh media. Cells were again quantified while re-plating and after they had been allowed to recover for an additional 24 h in chemical-free media.

To analyze cell cycle progression, $CD34^+$ cells (5×10^5) were plated and treated with the chemical concentrations indicated in the text for 24 h. Following treatment, cells were collected, washed with PBS and fixed in ethanol. Cells were then resuspended in PBS containing RNase and propidium iodide for 20 min in the dark. Subsequently, the cells were analyzed using a FACSCalibur flow cytometer to determine DNA content.

2.3. DNA breakpoint detection by LM-PCR

To detect DNA breaks, genomic DNA was isolated from human $CD34^+$ progenitor cells following treatment for 24 h and compared with untreated cells. Blunt-ended double-stranded DNA breaks were detected and quantified as described previously [9], with