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Surviving apoptosis: A possible mechanism of benzene-induced leukemia

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Abstract

The pathological consequences resulting from deregulation of the apoptotic program include cancer (too little apoptosis) or diseases of cell deprivation, such as Alzheimer's (too much apoptosis). We have identified an additional pathology whereby cells reaching the earliest stage of chromatin cleavage have the potential to suppress apoptotic execution and survive. One specific cleavage event associated with this process is restricted to a location within the mixed lineage leukemia (*MLL*) gene at 11q23. The site of cleavage is consistent with the location where large, ~50 kbp loops of supercoiled DNA are attached to the nuclear matrix. Cells modified by this process generate *MLL* translocations, as shown by inverse PCR, that survive for days to weeks but which have no known relationship with clinical disease. Using a specific approach, cells stimulated by anti-CD95 antibody, a potent stimulator of the apoptotic program, facilitated creation of the *MLL–AF9* fusion gene. Further, this rearrangement, which is commonly observed in patients with AML linked to exposure to cytotoxic agents, was efficiently transcribed in cells that were able to undergo cell division. These data are discussed in the context of benzene and benzene metabolite toxicity that impacts the process of apoptosis and is known to lead to leukemic disease.
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1. Introduction

As a result of industrialization and changes in lifestyle, the population at large has been exposed to a variety of agents that have subsequently been shown to trigger mutations and cancers. There is now substantial experimental and epidemiological evidence

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indicating that occupational exposure to benzene results in an increased risk of aplastic anemia, acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) [1–3]. Although there are studies showing the toxic effects of benzene, the mechanism(s) by which benzene causes leukemia still remain unclear.

1.1. Benzene biochemistry and disease

Benzene undergoes biological activation in the liver by cytochrome P450 (CYP450) forming benzene oxide, a portion of which is then converted to phenol by a non-enzymatic rearrangement. Phenol can be further metabolized to hydroquinone or catechol by CYP450. Once in the bone marrow, it is believed that hydroquinone and catechol are converted by myeloperoxidase to 1,4-benzoquinone and 1,2-benzoquinone, respectively [4]. These quinones are capable of inducing DNA strand breaks and inhibition of topoisomerase II, both of which, either singly or together, may cause chromosomal aberrations. Benzene exposure has been linked to the induction of both the myelodysplastic syndrome and acute myelogenous leukemia (AML) [5]. The leukemogenic pathway for benzene is similar to that observed in patients exposed to alkylating agents who show a protracted delay prior to AML [6]. Such leukemias usually involve loss or aberration of chromosomes 5 and/or 7. A more rapid leukemic course is observed, however, after treatment with agents that target topoisomerase II, including etoposide and some benzene metabolites [7]. These data suggest that this class of leukemias may also be triggered in those individuals exposed to benzene.

1.2. Topoisomerase II, benzene, and leukemia

A number of studies have evaluated the chromosomal effects of benzene, providing insights into the mechanisms of benzene genotoxicity. Recently it has been shown that benzene metabolites can inhibit purified preparations of human topoisomerase II [8–10]. DNA topoisomerases are nuclear enzymes, which induce transient breaks in DNA allowing the passing of one DNA strand through another [11–13]. DNA double strand breaks induced by topoisomerase II inhibitors can induce apoptosis of tumor cells contributing to the therapeutic effect [14]. Topoisomerase II is also thought to be a key intermediate in cases of leukemia in patients receiving topoisomerase II inhibitors and in leukemia in infants. In both cases, chromosome fusions involving the *MLL* gene at 11q23 are observed [15]. After exposure to topoisomerase II inhibitors such as etoposide or doxorubicin, *MLL* is translocated to least 40 different partner chromosomes, the most common being chromosomes 4, 6, and 9 [16]. Approximately 80% of infants with AML and ALL have chromosome translocations involving the *MLL* gene [13,17–19]. In the subset of AML patients linked to inhibitors of DNA topoisomerase II such as etoposide, characteristic reciprocal translocations involving 11q23 (*MLL*) have been observed. These have been suggested to result from site-specific DNA cleavage induced by the topoisomerase inhibitors [20,21]. In a study examining chromosomes 8 and 21 in workers exposed to benzene, reciprocal translocations were increased 15-fold in the peripheral blood cells of workers exposed to high concentrations [22]. Inhibitors of DNA topoisomerase II, such as etoposide and various benzene metabolites, have also been implicated as the causative agent in therapy-related leukemia and this argument is supported by the proximity of topoisomerase II binding sites (consensus sequences) within a region of *MLL* that is subject to frequent translocations. Drugs inhibiting topoisomerase II that are associated with *MLL* translocations in therapy-related AML, block the re-ligation of topoisomerase II-cleaved double strand breaks in cells, which ultimately results in cell death.

1.3. Benzene and apoptosis

The key suppressive effect of apoptosis in eliminating damaged and potentially neoplastic cells has been known and discussed for some time [23–26]. The relationship between benzene exposure and apoptosis however is controversial. In one recent report, it was demonstrated that exposure of NIH 3T3 cells to benzoquinone or hydroquinone inhibited apoptotic cell death induced by serum starvation or separation from an extracellular matrix (anoikis) [27]. This is an unusual response from a triggering agent that is most often considered as cytotoxic [28]. In a study of hydroquinone exposure in cells that were either myeloperoxidase rich (HL60) or deficient (Jurkat), it was demonstrated that hydroquinone induced apoptosis in both cell types. However, addition of a broad-spectrum caspase inhibitor, zVAD.fmk, was unable to suppress the very

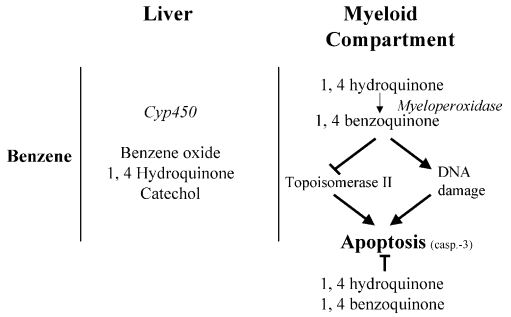

Fig. 1. Diagram showing the possible impact of reactive benzene metabolites with regard to DNA damage and apoptosis.

earliest signs of apoptosis, phosphatidyl leaflet externalization and cytoplasmic changes in the HL60 cell line [29]. One implication of these data is that hydroquinone processing by myeloperoxidase leads to products capable of activating an early, caspase-independent variant of apoptosis. These data suggest a complex and multifactorial impact of benzene exposure in terms of leukemia development; some of these issues are summarized in Fig. 1. It is clear however, that some, or all, benzene metabolites may affect the correct initiation or execution of apoptosis. In this manuscript, we highlight a novel pathway to leukemia involving a specific dysregulation of the apoptotic process that we have shown has the capacity to create leukemogenic translocations in cells with the capacity to survive.

2. Results and discussion

2.1. Apoptosis effectors and site-specific cleavage

We have provided evidence that the effector arm of the apoptotic program is capable of inducing genomic rearrangements linked to leukemia and that these may occur in cells that escaped normal apoptotic execution. It has been known from the work of a number of authors that cells exposed to pro-apoptotic signals undergo site-specific cleavage at 11q23 [20,30] (Fig. 2). Early studies by Stanulla et al. showed in both human and mouse cell lines that exposure to a variety of cytotoxic agents, including topoisomerase II inhibitors, produced specific cleavage within the breakpoint cluster region of *MLL* [20]. These data used a Southern blot as a readout of the cleavage involved, which was unable to determine the precise site of cleavage. We have refined the technique using ligation-mediated PCR which allowed a much greater degree of precision in determining the site of cleavage (Fig. 3). Here, a double-stranded DNA adapter is used to tag all DNA double strand breaks in the test system. Subsequently, primers to the *MLL* region of interest permit site-specific PCR. The size of the amplicon can then be used to determine the original cleavage location. Using this system, precision at the base pair level is possible, placing the cut site just 5′ to exon 12 of *MLL*. In the data shown, the activator of the apoptotic program was anti-CD95 antibody. This activates the extrinsic pathway to apoptosis, via a membrane receptor. Thus cleavage is restricted to the apoptotic program only and is not dependent on intrinsic damage to DNA, such as from benzene or topoisomerase II inhibitors. This approach has the advantage of restricting all sources of DNA damage to the effector component of apoptosis.

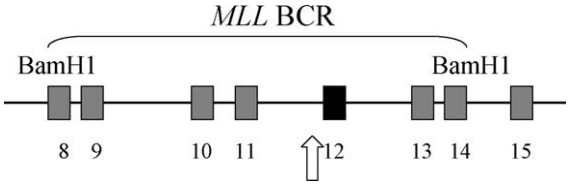

Fig. 2. Schematic representation of *MLL* cleavage by apoptotic stimuli within the breakpoint cluster region (BCR), defined by BamH1 restriction sites. Vertical arrow indicates the target for nuclease attack (site-specific cleavage) initiated within intron 11 of the *MLL* BCR.

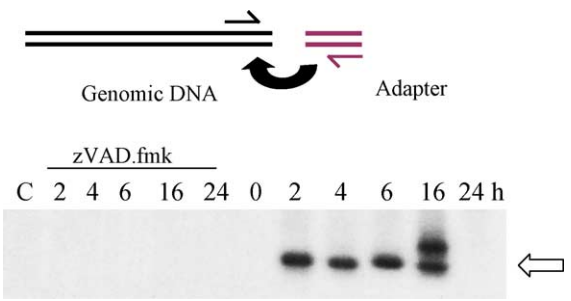

Fig. 3. Ligation-mediated PCR (LM-PCR) used to detect *MLL* cleavage in human TK6 cells triggered by 0.5 μg/ml of anti-CD95 antibody. Apoptotic-specific cleavage of *MLL* allows ligation of an artificial adapter to DNA breaks. This allows amplification of a PCR product specific to the adapter and *MLL* cleavage site. Such ligation-mediated PCR generates a 290 bp product (arrow). Addition of 20 μM pan caspase inhibitor (zVAD.fmk) blocks all cleavage.

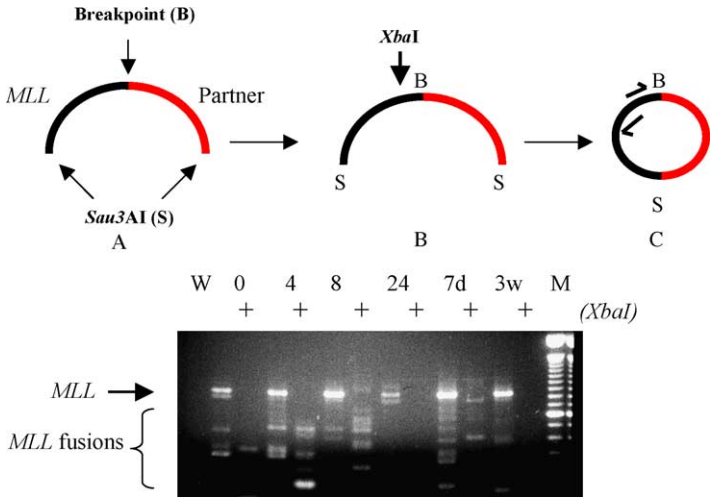

Fig. 4. Analysis of translocations by inverse PCR. Excision of a region surrounding the apoptotic cleavage site with a frequent-cutting enzyme (S) generates circular templates of known size. If the 5′ region of *MLL* has fused with a partner then the partner restriction site (S) will now be used to create a template of different size. In each case, the templates are platforms for inverse PCR generating the amplicons observed, giving a fixed size for genomic DNA (*MLL* arrow) and variable sizes dependent on the fusions that take place (*MLL* fusions) (see text for further details).

2.2. Apoptotic effectors and DNA damage processing

We have shown that cleavage at 11q23 induced by pro-apoptotic stimuli are targets for the NHEJ repair system, as seen by the rapid accumulation of the core NHEJ protein, DNA-PK, at the cut site [30]. Of perhaps greater significance, however, is the finding that cells cleaved at 11q23 by exposure to activators of the apoptotic program are targets for processing by the NHEJ system leading to ligation errors, in particular those that lead to translocations. To demonstrate such an effect, human cells of lymphoblastoid origin (TK6) were exposed to ionizing radiation or anti-CD95 antibody and returned to culture. For 3 weeks after exposure, DNA from the treated cells was extracted and analyzed for translocations at the specific *MLL* 11q23 cleavage site using inverse PCR [30] (Fig. 4). The protocol is shown diagrammatically in Fig. 4, but in essence the technique reveals translocations to unknown partners at the 11q23 breaksite by a two-step process. First, the region surrounding the target site is excised with a frequent-cutting enzyme, Sau3AI in this case. This removes short fragments of DNA from both the known genomic region (Sau3AI sites here are easily identified) and any unknown translocation breakpoints where the 5′ Sau3AI site of *MLL* remains, now attached to an unknown partner that will, however, contain its own Sau3AI site at some location. The second step requires each of such DNA fragments to be circularized (at low dilution to inhibit linking one fragment to another) at the common Sau3AI site and application of primers to the known *MLL* portion that extend in opposite directions. This latter step is the only peculiarity in that here, the primers extend in mutually opposed directions, rather than towards each other as in routine PCR. The net result is that the circularized fragments are amplified and *MLL* translocations may be detected by the size of circular fragments produced and verified by sequencing (Fig. 4). With this approach PCR products related to discrete translocations are visible for up to 3 weeks after exposure to the apoptotic reagent. This is far longer than expected if such rearrangements occurred in apoptosis committed cells. Treated TK6 cells regain full viability and their starting cell number 3 days after exposure to 0.5 μg/ml of the anti-CD95 antibody. Sequencing a sample of these translocations triggered by either irradiation or anti-CD95 antibody confirmed that they were indeed *MLL* translocations, but that none were linked to any patient-derived *MLL* translocation yet reported.

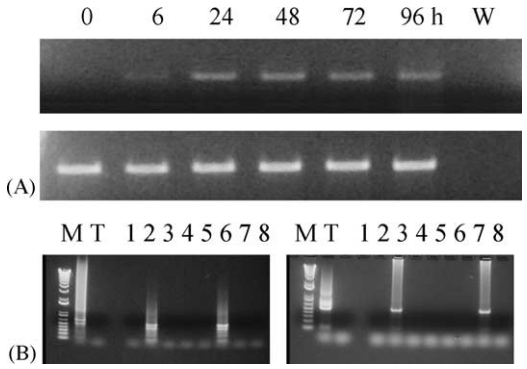

Fig. 5. Initiation of *MLL–AF9* fusion and transcription by apoptotic triggers in cells capable of division. (A) Top: kinetics of *MLL–AF9* production in TK6 cells after 0.5 μg/ml anti-CD95 antibody. *MLL–AF9* RT-PCR signal was observed from 6 h after exposure to antibody, W: water control. Bottom: GAPDH control for time series. (B) Here aliquots of TK6 cells treated as above were allowed to re-grow and subdivided into eight separate aliquots after 4 days. Genomic PCR spanning the *MLL–AF9* breakpoint followed by sequencing was then performed to detect the duplication of cell-specific, sequence identical, *MLL–AF9* breakpoints. Two such experiments are shown. M: markers; T: MM6 cell line positive for an *MLL–AF9* fusion gene (positive control).

2.3. Apoptosis–MLL–Leukemia

In order to link the idea of rescue from apoptosis to clinical experience, a screen was undertaken to search for the production of clinically relevant fusion genes in cells exposed to pro-apoptotic stimuli. The fusion gene *MLL–AF9* was selected as an example of a clinically relevant system as it is one of the more common *MLL* fusions observed, particularly after patient treatment with cytotoxic agents [31,32]. It was decided to assay for the presence of the *MLL–AF9* message as such an endpoint provides some confirmation of biological activity (transcription) and also may be available in multiple copies, so helping in detection. In these experiments, the apoptosis-specific trigger, anti-CD95 antibody, was again used with TK6 cells and the presence of *MLL–AF9* message determined by RT-PCR using the Titanium kit (Clontech, Palo Alto, CA). The results are shown in Fig. 5A. Surprisingly, only 6 h after stimulation a clear signal was observed that was confirmed to be the product of an *MLL*–AF9 fusion by sequencing of the PCR products cut from the gel [32]. Such a signal was observed for the length of this experiment (4 days). The fact that such a signal could be so rapidly identified raises a number of profound questions concerning the mechanism of fusion gene formation and its relevance to leukemia. Such a discussion should include the identification of *TEL–AML1* or *AML1–ETO* fusion genes in cord bloods at a frequency that is 100-fold greater than the risk of the corresponding leukemia [33]. One interpretation of these data is that cells trigger apoptotic effector activity creating fusion genes, but that most such modified cells die. The complexity of fusion gene biology continues after their creation in that the activity of *MLL–AF9* appears to be dose-dependent, inducing apoptosis at high expression levels but not at the low levels normally encountered in clinical material [34].

2.4. Is apoptosis survivable?

To be of biological relevance, cells containing such rearrangements at 11q23 or elsewhere must have the capacity to suppress apoptotic execution and survive. There are data supporting the view that cells can access the distal part of the apoptotic program and survive. A fraction of cells expressing the early indicator of apoptosis, Annexin V, are able to re-enter the cell cycle and continue to proliferate [35]. In addition, caspases-3, -6, and -7 were found to be enzymatically active in non-apoptotic-activated T cells [36]. Of these, caspase-3 may be activated by lysosomal enzymes in addition to apoptotic signaling pathways, suggesting that it may be activated under conditions other than terminal apoptosis [37]. Perhaps the most convincing data however are found in the nematode worm *C. elegans*, where cells morphogenetically predestined to die by apoptosis may recover from the apoptotic phenotype (nuclear morphology), in the absence of adjacent phagocytic cells [38]. In order to determine if cells containing 11q23 rearrangements can survive, we devised a procedure to detect the division of cells containing an *MLL–AF9* breakpoint. To be biologically active, genomic fusions between any two genes (but *MLL* and *AF9* here) would normally occur within intronic regions such that the translated fusion gene itself is identical. However, the very large regions of intronic DNA mean that it is extremely unlikely that individual chromosome fusions will occur at exactly the same point in both genes. Thus in an experimental setting, the induction and division of specific *MLL–AF9* fusions can be tracked by following the duplication of specific *MLL–AF9* genomic breakpoints. This was done in TK6 cells exposed to

anti-CD95 antibody, allowing the cells to fully recover both cell number and viability and then screening for specific breakpoint duplications in aliquots of the same population [32]. The results of two such experiments are shown in Fig. 5B. Sequencing of the predominant bands showed that they were indeed duplicated breakpoints, indicating that the cells containing them had divided. For the future, it is proposed to isolate such aberrant cells as viable clones and probe the mechanism controlling their survival.

3. Summary

The introduction of benzene into the body represents a broad attack on both DNA and key enzymes such as topoisomerase II. In addition, published data support a variable and unpredictable effect of benzene on cells programmed to die by apoptosis. Nevertheless, benzene exposure may lead to the generation of leukemic disease, making the dissection of the pathways involved an important undertaking. In this paper, we have presented one novel alternative involving deregulation of the apoptotic pathway that is consistent with most of the published data concerning benzene and leukemia. Thus, benzene and/or its metabolites may cause the inappropriate activation of apoptotic effector enzymes targeting the *MLL* gene at 11q23 while suppressing the final execution of the apoptotic program. The specificity of this model means that it is open to test, primarily by manipulation of apoptotic execution potential by either genetic or pharmacological means.

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