BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species – dependent DNA double-strand breaks

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ABSTRACT

The oncogenic BCR/ABL tyrosine kinase induces constitutive DNA damage in Philadelphia chromosome (Ph1)-positive leukemia cells. We find that BCR/ABL-induced reactive oxygen species (ROS) cause chronic oxidative DNA damage resulting in double-strand breaks (DSBs) in S and G2/M cell cycle phases. These lesions are repaired by BCR/ABL-stimulated homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) mechanisms. A high mutation rate is detected in HRR products in BCR/ABL-positive cells, but not in the normal counterparts. In addition, large deletions are found in NHEJ products exclusively in BCR/ABL cells. We propose that the following series of events may contribute to genomic instability of Ph1-positive leukemias: BCR/ABL → ROS → oxidative DNA damage → DSBs in proliferating cells → unfaithful HRR and NHEJ repair.

Running title: BCR/ABL oncogene promotes mutagenic DSBs repair
INTRODUCTION

The bcr/abl chimeric gene is derived from relocation of part of the c-abl gene from chromosome 9 to part of the bcr gene locus on chromosome 22 [t(9;22), Philadelphia chromosome=Ph1], and is present in most chronic myelogenous leukemia (CML) and a cohort of acute lymphocytic leukemia (ALL) patients 1,2. BCR/ABL exhibits two complementary roles in cancer: stimulation of signaling pathways that render leukemia cells independent of their environment and modulation of the response to DNA damage causing drug resistance 3,4. In contrast to normal cells, BCR/ABL-positive cells seem to be better equipped to survive genotoxic damage due to their enhanced ability to repair DNA lesions, prolonged activation of the G2/M checkpoint to provide more time for repair, and inhibited pro-apoptotic mechanisms 5. Clinical observations and experimental findings have shown that BCR/ABL stimulates also genomic instability, leading to mutations and chromosomal abnormalities 6-12. The accumulation of genetic errors is believed to be responsible for the transition from a relatively benign CML chronic phase (CML-CP) to the aggressive blast crisis phase (CML-BC). Aberrations in pathways regulating the DNA damage response in BCR/ABL-positive leukemia cells may contribute to this phenomenon.

DNA damage can directly result from genotoxic treatment or may simply occur as a consequence of the infidelity of genome duplication and/or genotoxic effects of compounds such as reactive oxygen species (ROS). ROS are generated as a normal byproduct of normal oxidative metabolism in eukaryotic cells and can cause damage to all molecules, including DNA 13. Single-strand oxidative DNA damage, including species such as 8-oxoguanine (8-oxoG), has been documented for many years 14. If not repaired, the damage can induce a number of deleterious effects including mutations 15 and double-strand breaks (DSBs) 16 leading to an elevated cancer risk 17.

BCR/ABL kinase-stimulated ROS 18 may exert a chronic genotoxic stress causing DNA damage in leukemia cells. Given the fact that mechanisms necessary for the repair of DNA
lesions might be altered by BCR/ABL \cite{10,19,22}, the probability of accumulating DNA errors seems to be much higher in BCR/ABL-positive cells compared with non-transformed cells, because more DNA damage occurs and, while overall repair capability is enhanced, the fidelity of repair mechanisms is compromised. A mutator phenotype may be essential for tumor cells to grow in various organs under diverse conditions and to resist anti-tumor treatments.

This work shows that BCR/ABL elevates the level of ROS resulting in numerous DSBs during genome duplication and division (S and G2/M phase). Unfortunately, the oncogene promotes unfaithful mechanisms of DSBs repair. We hypothesize that elevated levels of DSBs combined with unfaithful repair mechanisms may contribute to genomic instability and malignant progression of the Ph\(^+\)-positive leukemias.
METHODS

Cells
The murine growth factor-dependent myeloid cell line 32Dcl3 and BCR/ABL- or BCR/ABL[K1172R]-expressing clones 20 have been maintained in the presence of pre-tested optimal concentrations of IL-3 required to maintain their continuous proliferation. Bone marrow mononuclear cells from C57Bl/6 mice (mBMC) (The Jackson Laboratory, Bar Harbor, ME, USA) were infected with BCR/ABL-IRES-GFP or IRES-GFP retroviral particles as described 20. GFP+ cells obtained after sorting were cultured for 72h in the presence of pre-tested concentration of IL-3 and used for experiments. Bone marrow cells from CML patients (2 CML chronic phase and 2 CML blast crisis patients) and healthy donors (hBMC) described before 23 were obtained after informed consent. Mononuclear cells were cultured for 72h in the presence of pre-tested recombinant human IL-3 and stem cell factor (SCF) and CD34+ cells were isolated 24. Draa-40 25 and BCR/ABL-Draa-40 cells were cultured in DMEM supplemented with 10% FBS.

Inhibitors
Cells were treated with the following compounds: 2µM STI571 (Novartis Pharma AG, Basel, Switzerland), 0.2µM antioxidant pyrrolidine dithiocarbamate (PDTC), 100µM of the nitrone spin traps N-tert-butyl-α-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2µM wortmannin and 5mM caffeine (Sigma-Aldrich, St. Louis, MO, USA). STI571, PDTC, PBN, and DMPO were added for 48h, wortmannin and caffeine for 3h.

Comet assay
The comet assay was performed under alkaline conditions as described 26 with modifications 27. Comet tail moment was analyzed in 50 images, randomly selected from each sample, in duplicate.
experiments (total 100 images/sample); it positively correlates with the level of DNA breakage and alkali labile sites. The value of tail moment in particular sample was taken as an index of DNA damage. Since our measurement system was not calibrated, tail moment was presented in arbitrary units. Results represent mean±SEM (standard deviation of a mean). The data were analyzed using STATISTICA (StatSoft, Tulsa, OK, USA) statistical package. For the enzymatic treatment cells were drained in agarose and covered with an enzyme buffer (control) or the enzyme (1µg/ml of EndoIII or Fpg) in buffer, incubated for 30 min at 37°C as described, and the comet tail moment was analyzed as described above. The results obtained with a buffer only were subtracted from these obtained with an enzyme (an enzyme treatment usually increased the detection of DNA damage by ~2-fold.

**DNA fragmentation analysis**

Genomic DNA was isolated from 3–5x10⁶ cells using Dneasy Tissue Kit (Qiagen Inc., Valencia, CA, USA), and 2-3µg, was run (30V for 15 minutes followed by 70V for few hours) in 2.5% agarose gel containing ethidium bromide and photographed.

**ROS assay**

The levels of intracellular ROS were analyzed in cells growing in the presence of IL-3 using the redox-sensitive fluorochrome 2’,7’-dichloro-fluorescin-diacetate.

**HRR assay**

Draa-40 cells and BCR/ABL-Draa-40 cells have integrated one or two copies of the modified gene for GFP (DR-GFP, which contains an I-SceI site) as a recombination reporter and a fragment of the GFP gene (which contains a BcgI site) as a donor for homologous repair. A HRR event restores functional GFP expression (BcgI +), which is readily detected by flow cytometry.
NHEJ assay

NHEJ was measured in cell-free extracts as described\textsuperscript{30} with modifications\textsuperscript{31}. Briefly, $10^7$ cells were washed 3 times with ice-cold PBS, and cytoplasm lysis was performed on ice for 10 min in hypotonic buffer A: 10 mM HEPES, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, pH = 7.5; proteinase inhibitors: 2 $\mu$g/ml aprotonin, 2 $\mu$g/ml leupeptin, 0.5 mM PMSF, 0.5 mM DTT, 25 mM NaF, 0.2 mM NaVO\textsubscript{3}. Pellet of cells was spun down for 3 min in 6,000 $g$ at 4°C, and the cytoplasmic lysis procedure was repeated. A pellet containing nuclei was resuspended in buffer B: 20 mM HEPES, 25% glycerol, 500 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, pH = 7.5; proteinase inhibitors as in buffer A, and rapidly frozen and thawed three times (liquid nitrogen/37°C). Whole mixture was centrifuged at 30,000 $g$, 30 min., 2°C. Supernatant was dialyzed over night against buffer C: 25 mM Tris-HCl pH=7.5, 1 mM EDTA, 10% glycerol; proteinase inhibitors as in buffer A. Aliquots of nuclear protein samples were stored in -70°C. NHEJ reactions were performed in the following conditions: a mixture containing 10 $\mu$g of nuclear lysate, 1 mM ATP, 0.25 mM dNTPs, 25 mM Tris-Acetate pH=7.5, 100 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT was pre-incubated in 37°C for 5 min. The substrate - 200 ng of linear plasmid pBluescript KS+ (digested XhoI+XbaI to generate non-compatible 5' overhangs) was added to the reaction mix and incubated for 1 h at 37°C. The samples were then incubated with 1 $\mu$g of proteinase K in 65°C for 30 min. Products of NHEJ reaction were resolved in 0.5% agarose gel containing 0.5 $\mu$g/ml of ethidium bromide, scanned with Adobe Photoshop and analyzed by ImageQuant TL (Amersham Bioscience, Piscataway, NJ, USA).

Measurements of the DNA repair frequency and fidelity

Draa-40 parental and BCR/ABL-Draa-40 cells were transfected with I-SceI expression plasmid, and the frequency and fidelity of DSBs repair was examined as described by Pierce et al.\textsuperscript{32}, with modifications. Briefly, three days after I-SceI transfection, a fragment of the DR-GFP cassette
containing a putative DSB repair site was amplified by PCR using DR-GFP cassette-specific primers: forward (A), CAGCCATTGCCTTTTATGGT, and reverse, GCCTGAAGAACGAGATCAGC (B). The products were cloned into the TA cloning kit (Invitrogen, Carlsbad, CA) plasmid and transformed into the One Shot TOP10. The bacterial colonies containing BcgI site (HRR product), I-SceI site (replication product), or not containing BcgI and I-SceI sites (NHEJ product) were identified by Southern dot-blotting using the BcgI and I-SceI specific probes: GGTGGCATCGCCCTCGCC and GGTATTACCCCTGTATCCCTAGCCGA, respectively (hybridization conditions allowed detection of a sequence containing one mismatched base). The presence of NHEJ product in the BcgI/I-SceI double-negative colonies was confirmed by PCR (see below). Repair products were amplified from bacteria by PCR using the forward (C), AGGGCGGGGTTCGGCTTCTGG, and reverse (D), CCTTCGGGCATGGCGGACTTGA, primers to amplify the HRR products, and commercially available T3 and T7 primers to amplify the NHEJ products (primers spanning larger fragment of DR-GFP cassette were used here to reduce the chance of omitting more extensive deletions), and sequenced. HRR mutation frequency was calculated as: total number of mutated nucleotides/total number of sequenced nucleotides. Identical mutations detected in BcgI+ sequences and in I-SceI+ sequences (20 sequences/group analyzed) were subtracted from calculation to exclude the replication errors. An average gain/loss of the bases in the double-negative BcgI/-I-SceI- NHEJ sequences was determined by dividing the sum of acquired/deleted bases by the number of sequences. Twenty repair products (HRR and NHEJ)/experimental group were analyzed.

**Immunofluorescence**

Nuclear localization of the indicated proteins was detected by immunofluorescence as previously described. Briefly, cytospins from unsynchronized cells were fixed in PBS + 0.06% Triton X-100 + 4% formamide, washed in PBS + 0.06% Triton X-100, and blocked in washing buffer supplemented with 1% BSA. To perform cell cycle specific analysis cells were washed with PBS,
spun down and gradually re-suspended in ice cold fixing buffer (PBS with 0.06% Triton X-100, 4% formamide and 0.04% glutaraldehyde), and incubated on ice for 30min. Fixed cells were washed twice with PBS, re-suspended in 1ml PBS with 100µg of RNase DNAse-free (Roche, Mannheim, Germany), and incubated in room temperature for 15min. DNA was stained with propidium iodide as described 5. G0/G1, S, and G2/M cell populations were isolated by FACS and cyto spun. The cell cycle purity of sorted cell populations was confirmed by FACS. Cells were stained with first antibodies against γ-H2AX (Upstate Biotechnology, Lake Placid, NY, USA) and RAD51 (PC130, Oncogene Research Products, Cambridge, MA, USA), or Ku70 (AHP318, Serotec, Inc., Raleigh, NC, USA). The secondary antibodies conjugated with FITC (Alexa488) or RHO (Alexa568) were applied (Molecular Probes Inc., Eugene, OR, USA). Negative controls were performed without primary antibodies. DNA was counterstained with 4’,6´diamidino-2-phenylindole (DAPI). Specific staining was visualized with an inverted Olympus IX70 fluorescence microscope equipped with a Cooke Sensicam QE camera (The Cooke Co., Auburn Hills, MI, USA). At least 50 individual cells were analyzed/experimental group, as described before 10. All graphic adjustments were performed using Adobe Photoshop.

**Linker-ligation PCR (LL-PCR) assay**

Genomic DNA was purified by DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA, USA). The protocol for LL-PCR to detect broken-ended double-stranded DNA was described by Schlissel et al. 33 and followed with modifications. Briefly, the two oligomers BW-1 (GCGGTGACCCGGGAGATCTGAATTC) and BW-2 (GAATTCAGATC) were annealed to form a linker and stored frozen. Purified DNA (2µg) was incubated with Klenow polymerase and ligated to the linker. Ligated DNA (200ng) was used in a 50µl PCR assay containing 3pmol of the linker reverse primer (BW-1) and DR-GFP cassette-specific forward primer (AGGGCGGGGTTCGGCTTCTGG) or Na+/K+ ATPase-specific forward primer
(GCATTGACTTGGGCACGTGAC) and 2 units of Taq polymerase. After two rounds of PCR (2 x 20 cycles) the products were detected by Southern analysis using α-dCTP labeled DR-GFP probe (NarI-HpaI fragment of the DR-GFP gene) and γ-ATP labeled Na⁺/K⁺ ATPase-specific probe (GGCCTACGAGCAAGCTGAG oligomer), respectively.
RESULTS

*BCR/ABL elevates the levels of ROS to induce DSBs.*

A comet assay indicated that the presence of BCR/ABL kinase increased the level of DNA damage (strand breaks and abasic sites) on average by ~2.5-fold. Inhibition of the kinase by 48h incubation with 2µM STI571 \(^{34}\) or ROS by 48h incubation with the antioxidants: 0.2µM PDTC \(^{18}\), 100µM PBN and 100µM DMPO \(^{35}\), reduced the damage to the level detected in parental cells (Figure 1A). STI571 and the antioxidants inhibited intracellular ROS (data not shown). In agreement, gel electrophoresis also detected chromosomal fragmentation of the genomic DNA in BCR/ABL cells, but not in parental cells or in BCR/ABL cells treated with STI571 or PDTC (Figure 1B), implicating DNA breaks occurred specifically in BCR/ABL kinase-dependent, ROS-mediated manner. In order to directly detect oxidative damage of DNA we employed two enzymes, endonuclease III (EndoIII) and formamidopyrimidine-DNA glycosylase (Fpg), which converts oxidative lesions into gaps detectable by the comet assay. Addition of either EndoIII or Fpg to the comet reaction significantly increased the DNA damage in BCR/ABL cells relative to the parental cells; again the levels of oxidative DNA damage was abrogated by inhibition of BCR/ABL kinase with STI571 and reduction of ROS by PDTC (Figure 1C).

To characterize the DNA breaks in BCR/ABL cells, histone γ-H2AX was used to quantitate DSBs and to spatially localize them within the nuclei of cells. γ-H2AX seems to result from a specific ATM/ATR-dependent phosphorylation of histone H2AX on serine 139 in chromosomal regions encompassing megabase lengths of DNA adjacent to DSBs \(^{36-38}\). About 60% of the exponentially growing BCR/ABL-32Dcl3 cells contained γ-H2AX foci, in comparison to 20% of parental cells (Figure 2A). To confirm that this effect occurs also in primary cells, murine bone marrow cells (mBMC) were infected with BCR/ABL retroviral
particles. About 61% of BCR/ABL-positive growth factor-independent mBMC displayed γ-H2AX foci, in contrast to only 27% of normal counterparts (Figure 2A). Moreover, CML primary cell populations contained more γ-H2AX foci–positive cells than normal cells (17% and 2%, respectively; 4 CML patients and 3 normal donors were analyzed). The generally lower levels of γ-H2AX foci-positive normal human cells in comparison to murine counterparts may depend on the differences in basal oxidative damage levels; the former cells contain ~10-times less oxidized lesions than the latter ones 39.

The γ-H2AX foci were formed in response to DNA damage – dependent activation of ATM/ATR kinases, because wortmannin and caffeine (inhibitors of these kinases 40) dramatically reduced the number of foci (Figure 2A). Inhibition of BCR/ABL kinase by STI571 and blocking of ROS by PDTC diminished the percentage of γ-H2AX-positive BCR/ABL cells (Figure 2A). This result implicates BCR/ABL kinase-dependent, ROS-mediated mechanism leading to the elevated levels of leukemia cells bearing DSBs. In addition to the increased percentage of BCR/ABL-positive cells displaying γ-H2AX foci, these cells contained at least 2-times more foci/cell than non-transformed counterparts (Figure 2B). The percentage of γ-H2AX-positive cells containing ≤10 or >10 foci/nucleus was 3% and 97%, respectively, in BCR/ABL-32Dcl3 cells; in contrast to 75% and 25%, respectively, in parental 32Dcl3 cells.

To determine if γ-H2AX foci were associated with a particular cell cycle phase, exponentially growing 32Dcl3 parental cells and BCR/ABL-32Dcl3 cells were sorted on the G0/G1, S, and G2/M populations according to the DNA content. Analysis of cell cycle distribution of γ-H2AX –positive cells revealed that the majority of cells were in S and G2/M phase, but not in G0/G1 phase; BCR/ABL did not change their percentage distribution (Figure 2C).

Linker-ligation PCR (LL-PCR) was employed to detect DSBs in order to confirm the results obtained with the use of γ-H2AX foci as a marker of DSBs. We found two hotspots for
DSBs in the DR-GFP gene in BCR/ABL-Draa-40 cells; these DSBs were not detectable either in parental cells or in BCR/ABL cells if ROS was inhibited by PDTC (Figure 2D, upper panel). In addition, DSBs in the \( \alpha_1 \) subunit of the Na\(^+\)/K\(^+\) ATPase gene were detected by LL-PCR in BCR/ABL-32Dcl3 cells but not in parental 32Dcl3 cells (Figure 2D, lower panel). Again, inhibition of ROS by PDTC prevented detection of these DSBs in BCR/ABL cells. Combined analysis of the approximate length of LL-PCR products, position of the forward primers, and DR-GFP or Na\(^+\)/K\(^+\) ATPase gene sequences revealed multiple G/C-rich sequences at the predicted DSBs hotspots (Fig. 2D).

As expected, increased levels of ROS-dependent DSBs in DR-GFP gene in BCR/ABL cells were associated with increased levels of spontaneous HRR, as measured by detection of GFP+ cells after 8 weeks cell culture. HRR rate (estimated using the Luria-Delbruck fluctuation analysis with modifications \(^{41}\)) was 30x10\(^{-4}\) and 3.5x10\(^{-4}\) for BCR/ABL-Draa-40 cells and Draa-40 parental cells, respectively. BCR/ABL-Draa-40 cells incubated in the presence of PDTC displayed a reduced HRR rate, 11x10\(^{-4}\), in comparison to the untreated BCR/ABL-Draa-40 cells.

**BCR/ABL stimulates HRR and NHEJ to facilitate the repair of ROS-dependent DSBs.**

Since the majority of cells containing DSBs are in S and G2/M cell cycle phases, DSBs could be repaired by the two competing machineries: HRR and/or NHEJ \(^{42-44}\). The efficiency of HRR and NHEJ in BCR/ABL-positive and negative cells were examined using the specific tests.

A copy of the DR-GFP cassette containing inactivated GFP gene due to introduction of the unique I-SceI restriction site containing two stop codons and a truncated version of the gene was integrated into the genome of Draa-40 cells \(^{25}\). A DSB is generated in the GFP gene upon transient transfection with I-SceI expression plasmid, which could be repaired by HRR employing the truncated fragment of the gene as a template, thus producing a functional gene and hence GFP+ cells. Draa-40 cells containing DR-GFP cassette were transfected with
expression plasmids containing BCR/ABL wild-type, BCR/ABL[K1172R] kinase-inactive mutant (BCR/ABL[kin-]), or empty plasmid; the expression of BCR/ABL proteins was confirmed by Western analysis (not shown). Then these cells were transfected with I-SceI expression plasmid (and the β-galactosidase plasmid to control the transfection efficiency) and HRR frequency was measured by scoring the percentage of GFP+ cells. The presence of BCR/ABL active kinase, but not the kinase-dead mutant, caused ~4-fold increase of the percentage of GFP+ cells, implicating activation of HRR mechanism (Figure 3A).

pBluescript plasmid linearized by XhoI+XbaI digestion creating non-complementary 5’ overhangs was used as the substrate to assess the activity of NHEJ, which generates the products containing multimers of plasmid 30,45. The substrate was added to cell lysates from 32Dcl3 parental, BCR/ABL wild-type, and BCR/ABL[K1172R] kinase-inactive (BCR/ABL[kin-]) cells and NHEJ products were analyzed by agarose gel electrophoresis. BCR/ABL kinase activity was responsible for more than 5-fold increase of NHEJ activity; inactivation of the kinase by point-mutation (K1172R) reduced NHEJ by 2-fold (Figure 3B).

HRR and NHEJ reaction sites in the nuclei could be potentially visualized by double-immunofluorescence detecting co-localization of γ-H2AX foci with RAD51 or Ku70, respectively 46. As shown in Figure 3C about 50% of DSBs marked as γ-H2AX foci co-localize with RAD51 in parental and BCR/ABL-positive 32Dcl3 cells; similarly about 50% of the γ-H2AX foci co-localize with Ku70. This suggests that HRR and NHEJ are equally important for the repair of ROS-mediated “spontaneous” DSBs in BCR/ABL cells.

Parental cells treated for 48 h with 100 µM buthionine-sulfoximine (BSO), which inhibits γ-glutamyl-cysteine synthetase, thus increasing ROS by depleting glutathione pools 47, displayed enhancement of ROS (data not shown) and increased percentage (56±11) of γ-H2AX-positive cells (see group P in Figure 2A). However, elevation of ROS-mediated DSBs in parental cells was not associated with enhanced HRR (1.36±0.3% of GFP+ cells) and NHEJ
(5.8±1.8% of repair) in comparison to the untreated cells (see groups P in Figure 3A and 3B, respectively). Finally, about 10-20% of BSO-treated cells have been displaying an apoptotic signature (sub-diploid amount of DNA) after 72h of incubation. These effects were reversed by the anti-oxidant PDTC (data not shown), implicating ROS-dependence.

**BCR/ABL promotes mutagenic repair of DSBs**

Parental and BCR/ABL-Draa-40 cells were transfected with I-SceI expression plasmid to inflict a DSB in the reporter DR-GFP cassette. The primers spanning a fragment of the DR-GFP cassette containing the DSB site were used 72h later to amplify the repair products by PCR, which were then analyzed by Southern assay and sequenced to determine the frequency and fidelity of a repair mechanism (Figure 4A). The sequences with restored BcgI restriction site are considered HRR products; these without BcgI and I-SceI sites are identified as NHEJ products, and these with preserved I-SceI site are considered the replication products (DSB and repair did not occur).

A combined PCR-Southern analysis revealed that HRR and NHEJ consist of ~40% and 60% of DSBs repair products, respectively, and that BCR/ABL does not significantly change this proportion (Figure 4A, % of repair events), although it enhances both HRR and NHEJ activity (Figure 3A and 3B). HRR products obtained from parental cells were repaired faithfully (no mutations found), whereas the products from BCR/ABL-positive cells contained mutations (overall mutation rate: 6 x 10^{-3}) (Figure 4A, HRR branch). Analysis of the mutations revealed that almost 55% of mutations involved G:C → A:T transitions (40.4%) and G:C → T:A transversions (14.3%) – two of the most common mutations resulting from the oxidative DNA damage \(^\text{15}\). These mutations do not cluster near the DSB site (Figure 4B). Typical deletions or additions (range: +3 to -59 bp) were detected in NHEJ products in parental cells (Figure 4A, NHEJ branch). NHEJ products in BCR/ABL cells contained only deletions (range:
-2 to -533 bp); 35% of the products lost more than 100bp. On average, the presence of BCR/ABL induced a statistically significant ($p=0.01$) loss of DNA during NHEJ.
DISCUSSION

A mutator phenotype in cancer cells represents a major factor contributing to malignant progression of the disease \(^{48}\). In general, two aberrant functions may be attributed to genomic instability: enhanced DNA damage and compromised DNA repair mechanisms. DNA damage can occur as a result of the activity of endogenous compounds such as ROS and exogenous factors like radiation or genotoxic compounds \(^{49}\). The DNA repair machinery may be deregulated by either loss or gain of function \(^{50,51}\).

BCR/ABL cells treated with genotoxic agents displayed higher levels of DNA damage and aberrant DNA repair mechanisms \(^{5,19,20,22,52-54}\). These factors combined with impaired apoptotic response and prolonged activation of the G\(_2\)/M checkpoint can lead to genomic instability in BCR/ABL cells surviving the treatment \(^4\). However, we show here that even untreated BCR/ABL cells contained elevated levels of DNA lesions (including DSBs) in comparison to non-transformed cells. This effect was observed not only in cell lines but also in primary cells such as CML patient cells and murine bone marrow cells transformed by BCR/ABL, in agreement with others showing constitutive DNA damage in CML cells \(^{53}\). Recent report by Dierov et al. \(^{55}\) failed to detect spontaneous DNA damage shortly after the tetracyclin-induced BCR/ABL expression, suggesting that the effect may require more sustained presence of BCR/ABL.

We found that elevated levels of DSBs in BCR/ABL cells in comparison to normal counterparts resulted from ROS-dependent oxidative DNA damage. Stimulation of parental hematopoietic cell lines by a growth factor \(^{56}\) or BCR/ABL kinase \(^{18}\) resulted in elevation of ROS in comparison to the growth factor-starved parental cells. We cultured fully transformed growth factor-independent BCR/ABL cells and parental cells in the pre-tested optimal concentrations of IL-3 necessary to maintain continuous proliferation of the latter cells. In these conditions ROS was modestly enhanced (~2-fold) in the former cells (data not shown). This
effect may not be the sole factor contributing to the elevated oxidative damage in BCR/ABL cells. Degradation of the ROS-dependent oxidized nucleotides in the cytoplasm, their incorporation rate into DNA by polymerases, and/or the repair efficiency of the oxidized bases incorporated into DNA may be modified in BCR/ABL cells \(^{57}\). In addition, BCR/ABL may provide a necessary protection against apoptosis induced by these DNA lesions (e.g., DSBs) allowing accumulation of cells with damaged DNA \(^{58-60}\). This hypothesis is supported by our observation that elevation of ROS-induced γ-H2AX foci in parental cells treated with BSO was associated with apoptosis in some cells.

ROS can induce a variety of DNA lesions such as oxidative base damage (e.g., 8-oxoG) resulting from incorporation of the oxidatively modified nucleotides (e.g. 8-oxoGTP) into DNA by polymerases \(^{61}\). If not repaired properly these lesions may lead to mutations, formation of topoisomerase I – oxidized DNA cleavage complexes, DSBs at replication forks, and chromosome breaks detected on metaphase spreads \(^{15,16,62,63}\). Usually, oxidized bases are repaired by BER, which requires the activity of a DNA glycosylase and an AP endonuclease to generate gaps in a single-stranded DNA \(^{64}\). Such interruptions, if encountered by a replication fork, can cause DSBs \(^{62}\), which can be detected experimentally as γ-H2AX foci \(^{65}\). We hypothesize that ROS-mediated oxidative DNA lesions in BCR/ABL cells produce numerous DNA repair intermediates, which do not induce the G\(_1\)/S checkpoint in BCR/ABL cells \(^{5,66}\), resulting in DSBs at the replication forks. This speculation is supported by the finding that BCR/ABL cells containing numerous γ-H2AX foci (marker of DSBs) were detected almost exclusively in S and G2/M phase, but not in G0/G1 phase. This agrees with the previous observation that G\(_1\) cell cycle phase arrest prevented ROS-induced DNA strand breaks \(^{67}\). We were able to detect also a few cells with γ-H2AX foci in G\(_1\) phase, in agreement with the other report that ROS caused DSBs in serum starved G\(_1\) phase fibroblasts \(^{68}\).
We suggest that ROS-dependent DSBs present in BCR/ABL cells occurred in the regions containing multiple, 5-9bp long stretches of G/C. This supports a report that oxidative damage was predominantly detected in G/C-rich sequences \(^{69}\). Thus, it is tempting to speculate that the attempts to repair an extensive clustered oxidative damage in BCR/ABL cells may generate DNA lesions, which pose a serious obstacle for replication forks and result in DSBs. Lower levels of ROS in normal cells may cause limited DNA damage resulting in fewer DSBs, as suggested by detection of less \(\gamma\)-H2AX foci. Interestingly, LL-PCR did not detect DSBs in parental cells, which may simply reflect a limited sensitivity of the assay, or formation of \(\gamma\)-H2AX foci on the lesions different than DSBs. \(\gamma\)-H2AX foci may be detected also on single-stranded DNA at arrested replication fork \(^{70}\). We speculate that less abundant oxidative damage in parental and PDTC-treated BCR/ABL cells may cause stalling of a replication fork, which could be resolved by fork regression followed by reverse branch migration, without generation of a DSB. On the other hand, extensive oxidative DNA damage in BCR/ABL-positive cells may poise the mechanisms of fork regression/resetting, thus more often resulting in a DSB. In addition, suggested defect in the activation of intra-S phase checkpoint in BCR/ABL cells may also facilitate generation of DSBs \(^{55}\).

BCR/ABL cells have to develop protective mechanisms in response to ROS-dependent chronic genotoxic stress causing DSBs, e.g., prevention of caspase-3 activation and stimulation of DSBs repair \(^{4}\). HRR and NHEJ represent two major mechanisms of DSBs repair \(^{71}\). We show here that the majority of ROS-mediated DSBs occur in S and G2/M cell cycle phases, when both mechanisms are active and competing for a DSB substrate \(^{42-44}\). This work supports previous reports that BCR/ABL stimulated HRR \(^{20}\) and NHEJ \(^{52,53}\) reactions. Moreover, RAD51 and Ku70 co-localize with \(\gamma\)-H2AX foci (DSBs marker) with the same relative frequency, suggesting that HRR and NHEJ have an equal opportunity to be employed. Genetic analysis of the DSB repair products supports this hypothesis; about 40% and 60% of these products were
repaired by HRR and NHEJ, respectively. The modest shift toward NHEJ frequency, in comparison to that suggested by co-localization studies, may be explained by the observation that repair of some DSBs may be initiated by homologous invasion and completed by NHEJ event \(^{72}\). In summary, we hypothesize that both HRR and NHEJ play an important role in the repair of ROS-mediated DBSs in BCR/ABL cells.

We show that although enhanced, DSBs repair mechanisms are not faithful in BCR/ABL cells: mutations and large deletions were detected in HRR and NHEJ products, respectively. Since the mutations are rather scattered, not clustered near the DSBs induced by I-SceI or those detected by LL-PCR, the mechanisms responsible for ROS-dependent DSBs may be different from those causing HRR-mediated mutagenesis. We hypothesize that clustered oxidative damage in G/C-rich regions may cause DSBs, whereas DNA polymerase-mediated mispairing opposite to an oxidized base during HRR-dependent DNA replication may be responsible for the mutagenic effect. In addition, error-prone DNA polymerases, such as polymerase \(\beta \) [pol\(\beta\)], which expression is elevated in BCR/ABL cells \(^9\), may eventually replace other polymerases usually involved in DNA replication during HRR \(^{73}\). Interestingly, base misincorporations made during DSB repair in \(S. \) cerevisiae were not substrates for the mismatch repair machinery \(^{74}\). Therefore, mismatched bases incorporated to the HRR sites might not be removed efficiently, causing mutations in the recombination products. The molecular explanation for extensive degradation of DSBs preceding NHEJ in BCR/ABL cells is not known. Aberrantly regulated exonucleases like ExoI, Mre11 and Artemis could be suggested as potential mediators of this reaction \(^{75}\).

CML cells accumulate genetic abnormalities during the course of the disease \(^{76}\). The most frequently noticed involve additional chromosomes (Ph\(^1\), +8, +19), isochromosome i(17q) - associated with the loss of p53), reciprocal translocations (3;21 and 7;11) - associated with the expression of AML-1/Evi-1 and NUP98/HOXA9 fusion proteins, respectively, other translocations and inversions associated with AML/myelodysplasia (inv(3), t(15;17)), loss-of-
heterozygosity (LOH) at 14q32, homozygous mutations/deletions of pRb and p16/ARF, and mutations in p53 and RAS. The origin of these effects is mostly unknown, but errors in the repair of ROS-dependent DSBs described here can lead to the latter phenomena, especially to these involving intra-chromosomal deletions and point mutations. Therefore, we hypothesize that genetic instability leading to the malignant progression of Ph1-positive leukemias is associated with unfaithful repair of ROS-mediated DSBs.

In addition to BCR/ABL, myeloid cells transformed with other forms of the oncogenic ABL kinase, TEL/ABL and v-ABL, display the symptoms of ROS-dependent genotoxic stress (data not shown). Thus, unfaithful repair of ROS-dependent DSBs may represent a more universal mechanism of gaining a mutator phenotype in tumors caused by the ABL kinases, and possibly also in those expressing other fusion tyrosine kinases (FTKs: BCR/FGFR, TEL/ABL, TEL/JAK2, TEL/PDGFβR, NPM/ALK). Moreover, recent report by Heath & Cross showed that hematopoietic cells transformed by another FTK: ZNF198/FGFR1, which is associated with a disease that closely resembles BCR/ABL-positive CML, stimulate STAT5 to induce RAD51 and Bcl-xL expression, in accordance with our finding about other FTKs. Thus, cells transformed by FTKs seem to be prepared to handle increased levels of ROS-dependent “spontaneous” DSBs.
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FIGURE LEGENDS

Figure 1. BCR/ABL-induced ROS causes oxidative DNA damage. 32Dcl3 parental (P) and BCR/ABL-32Dcl3 cells (B/A) were cultured in the presence of IL-3; 2µM STI571, 0.2µM PDTC, 100µM DMPO and 100µM PBN were added for 48h when indicated. (A) Spontaneous DNA damage was detected by the comet assay. (B) Native genomic DNA was run through an ethidium-stained 2% agarose gel. (C) Oxidative damage to DNA was probed by Endo III endonuclease and Fgp glycosylase, and detected by the comet assay. Bars represent the enzyme-dependent increase of DNA damage over that detected in the undigested samples. (*p<0.05, in comparison to other experimental groups).

Figure 2. BCR/ABL-induced ROS causes DSBs in S and G2/M cell cycle phases. (A) γ-H2AX nuclear foci were detected by immunofluorescence in 32Dcl3 parental cells (P), BCR/ABL-positive counterparts (B/A), in cells treated with wortmannin + caffeine (Wt/Caff), in B/A cells treated with STI571 or PDTC, in normal murine bone marrow cells (mBMC) and BCR/ABL-transformed cells (mBMC-B/A), and in human BMC from healthy donors (hBMC) and CML patients (CML). The numbers indicate the percentage of cells with γ-H2AX foci (at least 5 foci/cell). Statistical significance (p<) of the results versus (v.) other experimental groups is shown. (B) Representative 32Dcl3 parental and BCR/ABL-32Dcl3 nuclei containing γ-H2AX foci. Only the foci co-localizing with DAPI are shown; nuclei borders are marked in blue. The numbers below show the mean number of foci/nucleus ± SD. (C) γ-H2AX foci were detected in 32Dcl3 parental (P) and BCR/ABL-32Dcl3 (B/A) cells isolated in G0/G1, S and G2/M cell cycle phases. Results show percentages of cell cycle distribution of the cells containing γ-H2AX foci. (D) DSBs (depicted by arrows) in the DR-GFP sequence (left panel) and Na⁺/K⁺ ATPase sequence (right panel) were detected by LL-PCR followed by Southern blotting in BCR/ABL-
positive cells (B/A), but not in parental (P) and PDTC-treated BCR/ABL-positive cells (B/A-PDTC). The G/C-rich stretches near the predicted DSB sites are listed.

**Figure 3. BCR/ABL-induced DSBs are repaired by HRR and NHEJ.** (A) HRR-dependent restoration of a functional GFP protein (GFP+ cells) after I-SceI-mediated induction of a DSB in Draa-40 parental, BCR/ABL-Draa-40 and BCR/ABL(kin-)-Draa-40 cells (*p<0.05, in comparison to other experimental groups). (B) NHEJ-mediated end-ligation of the XhoI+XbaI – digested plasmid substrate (monomers) by the lysis buffer (C), or cell lysates from 32Dcl3 parental (P), BCR/ABL-32Dcl3 (B/A) and BCR/ABL(kin-)-32Dcl3 (B/A (kin-)) cells, generating multi-plasmid products (dimers, trimers). The mean percentages of end-joined substrate ± SD are shown below (*p<0.05, B/A in comparison to BCR/ABL(kin-); p<0.01 B/A in comparison to P). (C) Co-localization (yellow) of γ-H2AX (red) with Rad51 or Ku70 (both green) in the representative 32Dcl3 parental (P) and BCR/ABL-32Dcl3 (B/A) nuclei, which borders are outlined in blue. The numbers indicate mean percentages of co-localization ± SD.

**Figure 4. BCR/ABL promotes unfaithful repair of DSBs.** Parental and BCR/ABL-Draa-40 cells were transfected with I-SceI expression plasmid to induce a DSB in the reporter DR-GFP cassette. (A) The scheme illustrates the consequences of I-SceI – induced DSB in DR-GFP: HRR restores BcgI restriction site (left branch) and NHEJ results in the loss of both I-SceI and BcgI sites (right branch). Repair products were amplified 72h later by PCR using primers A and B, cloned, and expressed in the competent bacteria. HRR (BcgI+) and NHEJ (BcgI-/I-SceI-) products were identified by Southern analysis (200 bacterial clones/group analyzed), amplified by PCR using primers C and D, or T3 and T7, respectively, and sequenced (20 sequences/group analyzed) to determine the repair mechanism (% of reparation events) and its fidelity (mutation frequency for HRR, and gain/loss of DNA base pairs for NHEJ). The mutations phenotype in the 725 bp
HRR products in BCR/ABL cells is shown in the bottom left diagram; gain/loss of DNA in the individual NHEJ products in Parental and BCR/ABL cells is shown in the bottom right diagram. (B) Individual mutations in the HRR products in BCR/ABL cells are shown (numbers indicate the number of mutations detected at the particular base). BcgI restriction site sequence is boxed.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species - dependent DNA double-strand breaks

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