BCR/ABL Inhibits Mismatch Repair to Protect from Apoptosis and Induce Point Mutations

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Abstract

BCR/ABL kinase–positive chronic myelogenous leukemia (CML) cells display genomic instability leading to point mutations in various genes including bcr/abl and p53, eventually causing resistance to imatinib and malignant progression of the disease. Mismatch repair (MMR) is responsible for detecting misincorporated nucleotides, resulting in excision repair before point mutations occur and/or induction of apoptosis to avoid propagation of cells carrying excessive DNA lesions. To assess MMR activity in CML, we used an in vivo assay using the plasmid substrate containing enhanced green fluorescent protein (EGFP) gene corrupted by T→G mismatch in the start codon; therefore, MMR restores EGFP expression. The efficacy of MMR was reduced ~2-fold in BCR/ABL-positive cell lines and CD34+ CML cells compared with normal counterparts. MMR was also challenged by N-methyl-N-nitro-N-nitrosoguanidine (MNNG), which generates O6-methylguanine and O4-methylthymine recognized by MMR system. Impaired MMR activity in leukemia cells was associated with better survival, accumulation of p53 but not of MMR system. Impaired MMR activity in leukemia cells was associated with better survival, accumulation of p53, p73, and activation of caspase 3, resulting in cell death. Ouabain–resistance test detecting mutations in the Na+/K+ ATPase was used to investigate the effect of BCR/ABL kinase–mediated inhibition of MMR on mutagenesis. BCR/ABL-positive cells surviving the treatment with MNNG displayed ~15-fold higher mutation frequency than parental counterparts and predominantly Gc→Ac and Ac→Gc mutator phenotype typical for MNNG-induced unrepairable lesions. In conclusion, these results suggest that BCR/ABL kinase abrogates MMR activity to inhibit apoptosis and induce mutator phenotype. [Cancer Res 2008;68(8):2576–80]

Introduction

BCR/ABL oncogenic tyrosine kinase transforms hematopoietic stem cells to induce chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (1). In addition to its capability to transform hematopoietic stem cells, BCR/ABL kinase can increase DNA damage and compromise the fidelity of DNA repair, causing genomic instability (2). Accumulation of genetic abnormalities is believed to be responsible for resistance to the ABL kinase small molecule inhibitors, such as imatinib, dasatinib, and nilotinib, and for transition of a relatively benign chronic phase (CML-CP) to the aggressive blast crisis (CML-BC).

Point mutations in BCR/ABL kinase sequence play a major role in resistance to small molecule inhibitors; overall, point mutations in the kinase domain of BCR/ABL have been detected in 50% to 90% of patients with acquired resistance to imatinib, including ~23% of the imatinib-naive patients (3). In addition, inactivating point mutations in p53 and Hb may contribute to the transition of CML-CP to CML-BC (4).

Our recent findings indicated that BCR/ABL-positive leukemia cells accumulate more DNA lesions due to enhanced levels of endogenous reactive oxygen species, resulting in point mutations (5, 6). Mismatch repair (MMR) system maintains genomic stability by removing mismatched bases from DNA and by inducing apoptosis in cells with excessive unrepairable DNA damage (7). Single-base mismatches are recognized by the MSH2-MSH6 heterodimer followed by MLH1-PMS2 heterodimer, which couples mismatch recognition step to downstream processes that include the removal of the mismatch from the nascent DNA strand, resynthesis of the degraded region, and ligation of the remaining nick. However, MMR also induces accumulation of p53 and p73, leading to activation of caspase 3 and apoptosis.

MMR activity in CML has been investigated indirectly by measuring microsatellite instability, which seems to reflect multiple replication errors because of the defective MMR (8). Microsatellite instability was not detected in the relatively benign CML-CP but was present in multiple loci in CML-BC (9). However, in CML-BC, microsatellite instability might be induced by secondary aberrations, thus not directly mediated by BCR/ABL.

Results presented here show that BCR/ABL kinase inhibits MMR, resulting in better survival and accumulation of point mutations, which may contribute to genomic instability in Philadelphia chromosome–positive leukemias.

Materials and Methods

Cells. 32Dcl3, BaF3 murine interleukin (IL)-3–dependent cell lines, and their BCR/ABL-transformed counterparts were described before (6). Cell lines were cultured in Iscove’s modified Dulbeco’s medium (IMDM) supplemented with 10% fetal bovine serum and pretested concentrations of WEHI-conditioned medium as a source of IL-3 required to maintain the proliferation of parental cells. CML-CP and CML-BC patient cells were obtained from the Stem Cell and Leukemia Core Facility of the University of Pennsylvania, Philadelphia, PA, USA, and the Institute of Hematology and Blood Transusions, Warsaw, Poland, after receiving informed consent.
CD34+ CML cells were obtained using human CD34+ selection cocktail (StemCell Technologies, Inc.). CD34+ normal bone marrow cells were from Cambrex Bio Science Walkersville, Inc. Primary cells were seeded in IMDM medium supplemented with 10% FBS and conditioned medium from baby hamster kidney–MKL cells (10%/v/v) as a source of stem cell factor and 5 ng/mL of human recombinant granulocyte-macrophage colony-stimulating factor and 2 ng/mL of human recombinant IL-3 (PeproTech, Inc.). Cells were precultured for 24 h with 40 μmol/L 6-O-methyl-2′-deoxyguanosine (Sigma Chemicals) to inhibit O6-methylguanine-DNA methyltransferase followed by the treatment with N-methyl-N-nitro-N-nitrosoguanidine (MNNG; Sigma).

Western analysis. Total cell lysates were prepared as previously described (10) and analyzed by Western blotting with the use of the following rabbit antibodies: anti-p73 (Santa Cruz Biotechnology), anti-p53 (Novoceastra), anti-active caspase 3 (Becton Dickinson Biosciences), and anti-tubulin (Calbiochem).

Ouabain resistance. Na+/K+ ATPase mutagenesis leading to ouabain resistance was examined as described before with modifications (6). Briefly, 106 cells growing in the presence of 1 μmol/L MNNG were plated in methylcellulose (StemCell Technologies) supplemented with WEHI-conditioned medium with or without 0.5 mmol/L ouabain (Sigma); colonies were counted after 7 d. Percentage of resistant cells was calculated as follows: number of colonies in the presence of ouabain × 100/number of colonies without ouabain. Ouabain-resistant single colonies were harvested, expanded, and murine α1 subunit of Na+/K+ ATPase was amplified by reverse transcription-PCR and sequenced.

Construction of T:G heteroduplex- and T:A homoduplex-containing plasmid substrates. Plasmid substrates used to calculate MMR activity were constructed as described by Lei et al. (11). Briefly, p5-1 construct (kindly obtained from Dr. LuZhe Sun, University of Texas Health Science Center, San Antonio, TX) was digested with ApaI and NruI (Promega). The larger fragment was isolated from agarose gel with Qiaex II (Qiagen, Inc.). Purified DNA was divided into three samples to generate a heteroduplex containing a T:G mismatch, homoduplex containing T:A match, and a negative control. To construct the T:G heteroduplex plasmid, we used EGF3-11 (5'-GGGATCCACCGGCCACCATGTTAGCACCATTGC-3') and EGF3-12 (5'-CGATTGCTCACCGTGGTGGCGACCGGTGGATCCCGGGCC-3') oligonucleotides. The homoduplex plasmid was constructed in the same way as the heteroduplex plasmid except that the oligonucleotide EGF3-12 was replaced with oligonucleotide EGF3-13 (5'-CGGATTCCTCAACATGTTGGCGGACTGCCGCGG-3'). For negative control, oligonucleotides were not used. Oligonucleotides were phosphorylated and annealed to form a double-stranded DNA fragment with T:G mismatch (heteroduplex) or T:A match (homoduplex). Two microliters of the oligonucleotide mixture were mixed with 1 μg of p5-1 larger fragment and 40 U of T4 ligase. After overnight ligation, DNA was digested with EcoRV, purified, and used for electroporations.

Electroporation. Parental cells, BCR/ABL-transformed cells, and BCR/ABL-transformed cells pretreated with 1 μmol/L imatinib (Novartis Pharma AG) for 24 h were mixed with T:G heterodimer or T:A homodimer and electroporated at 280 V and 900 F using a Gene Pulser II (Bio-Rad). CD34+ human cells were electroporated at 220 V and 900 F. Expression of enhanced green fluorescent protein (EGFP) was evaluated 48 h after electroporation by flow cytometry using EPICS XL (Beckman-Coulter, Inc.).

Calculation of relative EGFP expression. MMR efficiency in each cell line was measured with relative EGFP expression as described by Lei et al. (11). It was calculated according to the formula, relative EGFP expression = (M × IC50/N - M × IC20)/(C × IC50/N - C × IC20), where M, N, and C are the percentages of green cells for heteroduplex, negative control (mock transfection), and homoduplex transfection, respectively. I50, I20, and I0 are the mean fluorescence intensities of positive cells for M, N, and C, respectively. N × IC0 was omitted in the calculation when it was negligible.

Results

An assay using T:G mismatch–corrupted start codon of the EGFP gene was used here to quantitatively measure MMR activity in living cells (11). The mismatch can be corrected to T:A by MMR; therefore, MMR activity is determined by flow cytometry measuring the number of EGFP+ cells and the intensity of EGFP expression (Fig. 1A). To control the transfection and transcription/translation efficiencies, expression plasmid containing T:A match in EGFP start codon was applied. MMR activity was reduced ~2-fold in BCR/ABL-positive BaF3 and 32Dcl3 cells compared with parental counterparts (Fig. 1B). In addition, CD34+ cells from CML patients displayed inhibition of MMR activity compared with CD34+ cells obtained from healthy donors (Fig. 1B). The inhibitory effect of BCR/ABL on MMR was dependent on ABL kinase because pretreatment with imatinib restored MMR activity in leukemia cells to the levels observed in parental counterparts (Fig. 1B).
Recognition of a mismatch by MMR proteins usually induces apoptosis by accumulation of p53 and p73, leading to activation of caspase 3 (7). To determine if BCR/ABL kinase–mediated inhibition of MMR activity is associated with abrogation of p53/p73-caspase 3–apoptosis pathway, parental and BCR/ABL-transformed 32Dc13 cells were preincubated or not with O6-benzylguanine to inhibit O6-methylguanine-DNA methyltransferase and then treated with MNNG, a known mutagen and activator of MMR (12). O6-methylguanine-DNA methyltransferase may remove MNNG-induced base modifications before they are recognized by MMR; therefore, abrogation of its enzymatic activity eliminates the potential effect on MMR-dependent effects (13).

BCR/ABL-positive cells were resistant to MNNG compared with parental counterparts (Fig. 2A). Pretreatment with O6-benzylguanine did not significantly affect the sensitivity of leukemia and normal cells to MNNG, suggesting that O6-methylguanine-DNA methyltransferase does not play a major role here. As expected, MNNG-treated BCR/ABL-positive cells displayed robust time-dependent accumulation of p73 compared with the modest effect detected in parental cells (Fig. 2B; ref. 14). Interestingly, MNNG caused stimulation of p73 and activation of caspase 3 in parental cells but not in BCR/ABL-transformed counterparts (Fig. 2B). In concordance, immunofluorescence studies showed MNNG-induced accumulation of p73 in the nuclei of parental but not BCR/ABL-positive cells (Fig. 2C).

Cells surviving the initial treatment and subsequently growing in the presence of 1 μmol/L MNNG were plated in methylcellulose in the presence or absence of 0.5 mmol/L ouabain. Ouabain, a Na+/K+ ATPase inhibitor, is toxic for cells, but amino acid substitutions due to point mutations in the Na+/K+ ATPase 3-subunit of the enzyme may cause resistance to the toxin (15). The percentage of BCR/ABL-positive clones resistant to ouabain was ~15-fold higher than that of parental clones (Fig. 3A). The same numbers of MNNG-resistant parental and BCR/ABL-positive cells were used for clonogenic assay, and the results were expressed as percentages of clonogenic cells to exclude the possibility that increased mutation rate of BCR/ABL-positive cells simply reflect their increased survivability. Point mutations were detected in the α1 subunit of Na+/K+ ATPase gene in the resistant clones with expected prevalence of G:C→A:T and A:T→G:C transitions (Fig. 3B).

**Discussion**

Using the reporter plasmid where MMR can restore functional EGFP gene by repairing T:G mismatch in the start codon (11), we showed that BCR/ABL kinase inhibits MMR activity. T:G mismatch was chosen because it might lead to T→C transition detected in ~25% of bcr/abl mutations in CML stem cell–enriched populations and be responsible for imatinib-resistant BCR/ABL kinase variants, for example, Y253H and M351T (16).

T:G mismatch could be repaired by MMR or a mismatch-specific thymidine DNA glycosylase (TDG; ref. 17). However, only MMR-dependent pathway would create T:A match if the G is located in the antisense strand, restore the EGFP start codon, and induce EGFP expression. Repair of T:G mismatch by TDG would create the C:G fixed mutation in the EGFP start codon that prevents expression of the protein.

Reduced MMR activity reported in leukemia cells was usually associated with down-regulation/loss of at least one of the MMR proteins (18). However, expression of MSH2, MSH6, MLH1, and PMS2 proteins was not inhibited by BCR/ABL kinase in hematopoietic cell lines (Supplementary Fig. S1A) and CML-CP and CML-B cells (Supplementary Fig. S1B).

To study the biological effects of a negative influence of BCR/ABL kinase on MMR, cells were challenged with MNNG, a mutagen usually used to study MMR in vivo (12). MNNG produces a variety of structurally diverse alkyl-DNA lesions, but O6-methylguanine and O4-methylthymine are among the most frequent. These lesions are very efficiently recognized by MMR, which triggers signaling to apoptosis and excision removal of these lesions (7, 12). If not

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**Figure 2.** BCR/ABL-positive leukemia cells do not accumulate p73 in response to MNNG. A, parental 32Dc13 cells (P) and BCR/ABL-32Dc13 cells (B/A) were preincubated or not with O6-benzylguanine and treated with MNNG for 6 h. Cell viability was assessed 42 h later by trypan blue exclusion test. B, total cell lysates were obtained from cells pretreated with O6-benzylguanine and treated with 1 μmol/L MNNG, and analyzed by Western blotting. C, cells were treated (+) or not (−) with 1 μmol/L MNNG for 12 h. p73 was detected by immunofluorescence on the cytospins; DNA was counterstained by 4′,6-diamidino-2-phenylindole. Panels show individual cells.
repaired, replication may generate O6-methylguanine:T and O4-methylthymine:G mispairs, eventually resulting in point mutations.

BCR/ABL-positive leukemia cells proliferating in the presence of MNNG did not display reduced expression of MMR proteins (Supplementary Fig. S1A and C). Thus, it is unlikely that any effect of BCR/ABL kinase on MMR was due to selection of clones with low levels of MMR proteins. Interestingly, the assembly of MLH1-PMS2, but not MSH2-MSH6 heterodimer on MNNG-induced DNA lesions, was abrogated by BCR/ABL kinase in cell lines and CD34+ CML primary cells (Supplementary Figs. S2 and S3). These observations suggest that BCR/ABL kinase induces posttranslational modifications of MMR proteins affecting their physiologic response to mismatched bases.

Using MNNG as a mutagen inducing MMR-sensitive DNA lesions and ouabain resistance as an indicator of mutagenesis in Na+/K+ ATPase encoding resistance to ouabain. A, the percentage of ouabain-resistant cells (percentage of ouabain-resistant cells) was determined by clonogenic assay performed in the presence or absence of 0.5 mmol/L ouabain; P value of B/A (BCR/ABL-positive cells) compared with parental (P) group. Results represent four independent experiments performed in triplicate. B, mutation phenotype in mouse α1 subunit of Na+/K+ ATPase is shown.

In summary, reduced MMR activity in CML cells may be directly responsible for generation of point mutations in bcr/abl kinase sequence and in other genes such as p53 and Rb, causing resistance to small molecule inhibitors and malignant progression of the disease (2). This hypothesis is supported by the report that imatinib-resistant BCR/ABL mutants were recovered from MMR-deficient bacteria (20). In addition, N-ethyl-N-nitrosourea, which induced mutagenesis in MMR-deficient cells, generated imatinib-resistant BCR/ABL mutants in experimental conditions (21). Moreover, Msh2+/− bone marrow cells infected by Abelson murine leukemia virus acquire transforming phenotype and p53-inactivating point mutations with much higher frequency than Msh2+/− counterparts (22). It could be also postulated that impaired MMR could facilitate point mutations acquired during unfaithful nucleotide excision repair and homologous recombination repair in BCR/ABL-positive leukemias (5, 23). Thus, the precise effect of BCR/ABL kinase on MMR pathways requires further studies.

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