Expression of Oncogenic Kinase Bcr-Abl Impairs Mitotic Checkpoint and Promotes Aberrant Divisions and Resistance to Microtubule-Targeting Agents

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Abstract
Recent findings showed that BRCA1, in addition to its role in DNA damage response, acts as an upstream regulator of genes involved in the mitotic checkpoint regulation, thus protecting against promotion of aberrant divisions and aneuploidy. Moreover, there is also an indication that the BRCA1 protein is downregulated in chronic myeloid leukemia (CML) patients. We have investigated a possible functional relationship between BRCA1 and mitotic checkpoint competence in cells with the same genetic background expressing different levels of Bcr-Abl, an oncogene responsible for CML. Herein, we show that Bcr-Abl strongly downregulates the BRCA1 protein level, which is partially reversed on treatment with imatinib, an inhibitor of Bcr-Abl tyrosine kinase. Bcr-Abl leads to decreased expression of genes involved in the mitotic checkpoint activation—Mad2, Bub1, Bub3, and BubR1, resulting in mitosis perturbances, weakened mitotic checkpoint function, and mitotic slippage after nocodazole treatment. Furthermore, high Bcr-Abl–expressing cells showed also postmitotic checkpoint dysfunctions and inability to effectively arrest in the 4NG1 phase of the cell cycle, which was associated with limited p21 induction. These observations had significant biological consequences, as we found a high level of improper divisions, chromosomal missegregation, and generation of polyploid cells on mitotic checkpoint prolonged activation. Additionally, Bcr-Abl–expressing cells showed resistance to death activated by spindle defects, reversed by imatinib. Our study presents new facts and supports the hypothesis concerning the mutator nature of Bcr-Abl itself. The functional interaction between Bcr-Abl and mitosis dysfunctions, due to compromised mitotic checkpoints, may have important implications for the generation of aneuploidy and CML progression.

Introduction
Expression of the Bcr-Abl kinase causes chronic myeloid leukemia (CML), and its overexpression has been associated with disease transition from a chronic phase to more acute stages (1). Genomic instability is common in blast crisis and may contribute to malignant progression and resistance to cell death (2–5). It is believed that the genomic instability observed in CML is a result of the mutator nature of Bcr-Abl, which leads to an increased rate of mutations. However, it has also been proposed that Bcr-Abl can affect proteins responsible for cell cycle control. In this way, it would act as an amplifier of the genetically unstable phenotype (6). One study showed that BRCA1 protein was downregulated in Bcr-Abl–expressing hematopoietic cells (7). Apart from its well-known role in DNA repair, BRCA1 acts also as a factor-regulating gene expression for orderly mitotic progression, namely those involved in the spindle assembly checkpoint (SAC), also called the mitotic checkpoint (8, 9). SAC ensures accurate segregation of chromosomes during mitosis and delays the transition to anaphase in the presence of unattached or unaligned chromosomes (10). This delay is achieved through inhibition of the anaphase promoting complex, thereby preventing sister chromatid separation and cyclin B1 degradation. Several evolutionarily conserved proteins, including BubR1, Bub1, Bub3, Aurora B, Mad1, Mad2, and others, are required for effective checkpoint function (11, 12). When the SAC functions properly, prolonged incubation with microtubule-targeting agents causes metaphase arrest leading to mitotic catastrophe and eventual cell death. Cells with a compromised mitotic checkpoint undergo mitotic slippage associated with premature cyclin B1 degradation, chromatin decondensation, and arrest at a postmitotic checkpoint (tetraploid checkpoint) to prevent polyploidization. A defective mitotic checkpoint has been proposed to...
contribute to chromosomal instability. Our recently published data showed a high level of polyploidy in Bcr-Abl-expressing cells on depletion of survivin, one of the key proteins involved in control of mitosis (13). Generation of polyploids took place also in K562 cells after mitotic checkpoint impairment. Both observations suggested that expression of Bcr-Abl led to some dysfunctions in the mitosis regulation. Bearing in mind the proposed role of BRCA1 in the SAC, here we investigated the influence of Bcr-Abl expression on the BRCA1 level as well as competence and efficacy of the mitotic checkpoint. We used a cell line model described previously, in which cells with the same genetic background differ in the level of Bcr-Abl (14, 15). The model is based on the mouse progenitor 32D cells, with wild-type p53, stably transfected with p210 Bcr-Abl. We used two clones, expressing low and high levels of Bcr-Abl, referring to the chronic phase and blast crisis of CML, respectively.

We found that Bcr-Abl caused strong downregulation of BRCA1 and decreased the expression of the mitotic checkpoint components. Our data show that Bcr-Abl expression limits the ability of the mitotic checkpoint to undergo activation and compromises the postmitotic checkpoint, which normally prevents polyploidization. A consequence of these dysfunctions is the generation of aneuploid cells on prolonged checkpoint failure and resistance to chemotherapeutics called microtubule poisons. According to our knowledge, this link between Bcr-Abl expression and deregulation of the mitotic and postmitotic checkpoints has not been documented before.

Materials and Methods

Cell culture and treatment. 32D mouse progenitor cells and Bcr-Abl–expressing C2 and C4 clones were provided by Dr. S.L. McKenna. Cells were maintained as described previously (13) and treated with nocodazole (Sigma) at a concentration of 100 ng/mL or paclitaxel (Sigma) at a 100 nmol/L concentration. Cells are routinely tested for Mycoplasma and interleukin-3 (IL-3) dependence, and morphology is checked by microscope. Imatinib was kindly provided by the Pharmaceutical Research Institute in Warsaw and used in a concentration of 5 μmol/L.

Assessment of cell viability. Cell viability was assessed by the propidium iodide (PI) exclusion assay on a FACSCalibur flow cytometer (Becton Dickinson). Before the analysis, cells were resuspended in PBS with 50 μg/mL PI (Sigma).

Bromodeoxyuridine assay. For bromodeoxyuridine (BrdUrd) assay, after appropriate time of incubation with nocodazole, BrdUrd (Sigma-Aldrich) was added to the culture medium to a final concentration of 10 μmol/L for 20 minutes. Then, 1 × 10^6 cells were collected and washed with PBS containing 0.1% Tween 20 followed by a 30-minute incubation with 2 mol/L HCl at room temperature. After washing with 0.1 mol/L sodium tetraborate (Sigma-Aldrich) and twice with PBS–0.1% Tween 20, samples were incubated for 1 hour at room temperature with primary antibody against BrdUrd (Becton Dickinson), washed in PBS–0.1% Tween 20, and incubated with a secondary, phycoerythrin-conjugated antibody (Becton Dickinson). After washing, cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Nuclear morphology. To analyze nuclear morphology, cells were fixed in 70% ethanol, stained with 50 nmol/L 4′,6-diamidino-2-phenylindole (DAPI) dye (Sigma), and observed under a fluorescence microscope (Nikon) with a ×40 objective.

Mitotic index. Mitotic index was estimated by immunostaining using the anti-phosphorylated Ser/Thr-Pro MPM-2 antibody (Upstate) and the Alexa Fluor 488 secondary antibody (Invitrogen). For the mitotic index in polyploid population, cells were stained with MPM-2 as above, followed by staining for nuclear morphology with DAPI. Samples were analyzed on an iCys Research Imaging Scanning Cytometer (Compucyte).

Cell lysis and Western blotting. Whole-cell protein extracts were prepared with radioimmunoprecipitation assay buffer (13). Membranes were incubated with primary antibodies specific to p53 (1C12; Cell Signaling), Bub3 (BD Transduction Laboratories), BRCA1 (R&D Systems), p21 (C-19), cyclin B1 (H-433), Mad2 (FL-205), or Bub1 (H-300)—all from Santa Cruz Biotechnology. Anti-β-actin (Ab-1) and c-Abl antibodies were obtained from Calbiochem.

Immunoprecipitation and cyclin-dependent kinase 1 kinase assay. The cells were lysed with modified radioimmunoprecipitation assay buffer (13). Lysates (150 μg) were precleared by incubation with protein A/G plus agarose immunoprecipitation reagent (Santa Cruz Biotechnology, Inc.) for 30 minutes at 4°C with rotation. The supernatants were incubated with agarose-conjugated mouse monoclonal anti-cdc2/cyclin-dependent kinase 1 (cdk1) antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C with rotation. The beads were washed thrice with radioimmunoprecipitation assay buffer and assayed for histone H1 kinase activity (Upstate Biotechnology) according to the manufacturer protocol. Briefly, beads were suspended in kinase buffer containing 0.02 mg histone H1, 112.5 μmol/L ATP, and 5 μCi of [γ-32P]ATP and incubated at 30°C for 10 minutes. The phosphorylated substrate was then separated from the residual [γ-32P]ATP using P81 phosphocellulose paper and washed thrice in 0.75% phosphoric and once in acetone. The sample radioactivity was quantified using a scintillation counter (Beckman Coulter). Presented results reflect three independent experiments.

RNA interference. BRCA1 siRNA oligonucleotide corresponding to nucleotide sequence 5′-CGAUGUUC-CUUGGAUAACA-3 (Silencer Select Predesigned siRNA; ID s63079) was obtained from Ambion. siRNA was resuspended in RNase-free water and stored at −20°C. Transfection of siRNA was carried out using electroporation (Bio-Rad Gene Pulser Xcell Total System). Three million cells in 600 μL RPMI 1640 were incubated with siRNA.
(final concentration, 60 nmol/L) in a 0.4-cm cuvette for 5 minutes on ice before electroporation (260 V, 950 μF). After an additional 5-minute incubation on ice, cells were resuspended in 3 mL RPMI 1640 supplemented with glutamine and 10% FSC without antibiotics. Antibiotics (1% penicillin/streptomycin) were added at 6 hours after electroporation. All measurements were done at 24 hours after transfection.

**DNA content analysis.** Cell cycle distribution, polyploid formation, and apoptotic sub-G₁ were analyzed by flow cytometry. Cells were suspended in the Nicoletti buffer [0.1% sodium citrate (pH 7.4), 0.1% Triton X-100, 50 μg/mL PI]. Cell cycle analysis was done using the ModFit program (Becton Dickinson).

**Centrosome staining.** Cells were fixed, permeabilized, and incubated with the primary antibody against γ-tubulin (Sigma) at 4°C overnight and secondary anti-mouse horseradish peroxidase–conjugated antibody (Dako). For nuclear morphology observation, slides were stained with 50 nmol/L DAPI and analyzed using a fluorescence microscope with a ×40 objective.

**Quantitative real-time reverse transcription–PCR.** Total RNA was extracted using Tri Reagent (Molecular Research Center). The DNA-free DNase treatment and removal reagents (Ambion) were used to remove DNA contamination. cDNA was synthesized using 2 μg of RNA and Omniscript RT kit (Qiagen). Quantitative reverse transcription–PCR (RT-PCR) was done using a LightCycler 2.0 System (Roche Diagnostics). All reactions were done in a volume of 20 μL with 10× diluted cDNA as a template, 2 μL of LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics), 3 mmol/L MgCl₂, and forward and reverse primers each at 20 μmol/L. The annealing temperature was 50°C for Bub1, Bub3, and Mad 2; 53°C for BubR1; and 55°C for actin. The primer sequences are listed in Table 1. The obtained data from three independent experiments were analyzed with Light Cycler3 Run version 5.32. Levels of mRNA for the investigated genes were adjusted to the value obtained for β-actin.

**Results**

**The level of BRCA1 and spindle checkpoint components are reduced in Bcr-Abl–expressing cells.** To address the relationship between Bcr-Abl expression and spindle checkpoint competence, we analyzed the level of the BRCA1 protein and expression of the spindle checkpoint components in parental 32D cells and their counterparts with low (C2 cells) or high (C4 cells) Bcr-Abl level. We found that BRCA1 was strongly downregulated by Bcr-Abl expression (Fig. 1A). The difference was not changed by the treatment with MG132—an inhibitor of the proteasome (not shown). Imatinib treatment partially reversed this effect, indicating tyrosine kinase dependence of the BRCA1 downregulation. As BRCA1 had been proposed to affect the spindle checkpoint at the level of gene expression, we analyzed the expression of several spindle checkpoint genes (Mad2, BubR1, Bub1, and Bub3) at the mRNA (Fig. 1B) and protein (Fig. 1B) levels. An inverse relationship between the level of Bcr-Abl and expression of those genes was found at both levels. Treatment with MG132 did not reverse differences in the protein levels observed in control and treated cells (not shown), confirming that the difference is a result of Bcr-Abl–mediated decrease of protein synthesis. To confirm the regulatory link between BRCA1 and the expression of mitotic checkpoint components, which we proposed as a possible mechanism, we silenced BRCA1 expression in 32D cells, using RNA interference. At 24 hours after transfection with siRNA, BRCA1 expression decreased below 40% of the control level (Fig. 1C) and protein was barely detectable (not shown). At that time, we did quantitative analysis of the expression of Mad2, BubR1, Bub1, and Bub3 genes. Data obtained from four independent experiments (presented in Fig. 1C) show significant decrease in expression of all examined genes, indicating direct regulatory role of BRCA1. If Bcr-Abl expression is directly responsible for the decrease of mitotic gene expression, inhibition of Bcr-Abl tyrosine kinase activity by imatinib should lead to the reversed phenotype. To prove that tyrosine kinase activity of Bcr-Abl influences mitotic checkpoint genes expression, we treated 32D, C2, and C4 cells with imatinib for 6 hours and did real-time RT-PCR studies of gene expression (Fig. 1D). As shown previously, at that time, the increase in BRCA1 was already detected (Fig. 1A). In 32D cells, as we expected, any significant change in the level of expression was detected after 6 hours with imatinib. C2 and C4 cells showed increased expression of all examined genes, which confirmed previously obtained data indicating that Bcr-Abl negatively regulates expression of the mitotic checkpoint components. Altogether, these data

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show the direct role of BRCA1 as an upstream regulator of mitotic checkpoint gene expression in a Bcr-Abl-dependent manner.

**Bcr-Abl–expressing cells escape metaphase arrest and undergo mitotic slippage on nocodazole treatment.** To determine whether Bcr-Abl expression affects mitosis, we studied the competence of the mitotic checkpoint by using nocodazole, which disturbs microtubule dynamics, typically leading to mitotic arrest. Cell cycle analysis of 32D, C2, and C4 cells showed that, after 24 hours of nocodazole treatment, ~70% to 80% of all cells were arrested in G2-M phase of cell cycle, indicating...
Figure 2. Cells expressing Bcr-Abl escape mitosis on nocodazole treatment. A, cell cycle analysis estimated by DNA content measurement by flow cytometry. Cells were treated with 100 ng/mL nocodazole for 24 h. Ten thousand cells were analyzed for each sample. Typical histograms for three separate experiments are presented. B, mitotic index estimated on nocodazole treatment by MPM-2 staining. Three hundred cells were counted in three separate experiments. All data are presented as mean ± SEM. **, $P < 0.005$, versus 32D cells, by Student’s t test. C, nuclear morphology of untreated and nocodazole-treated cells (×400). Cells were incubated with nocodazole, fixed, and stained with DAPI. Arrow, cell with mitotic catastrophe symptoms; arrowheads, cells with decondensed chromosomes. D, left, cyclin B1 level analyzed in control and nocodazole-treated 32D, C2, and C4 cells. β-Actin as loading control. Right, the level of Cdk1 activity estimated in control and nocodazole-treated cells. Values were calculated relatively to control defined as 1.0; data are presented as mean ± SEM from three independent experiments.
arrest with 4C DNA content (Fig. 2A). To verify whether cells were indeed arrested in mitosis, we estimated the mitotic index by MPM-2 epitope detection, which specifically stains cells in mitosis. The amount of mitotic cells increased with time, and the highest percentage of mitosis-arrested cells (∼40–45%) was observed in all three cell lines after 12 hours (Fig. 2B). Thereafter, in the C2 and C4 populations, the fraction of MPM-2-positive cells decreased dramatically, and after 24 hours, the amount of MPM-2 did not exceed 15%. We can conclude that, at 24 hours, C2 and C4 cells still possessed 4C DNA, which is shown in Fig. 2A, but already escaped mitosis—they became MPM-2 negative (Fig. 2B). In contrast, the population of MPM-2—positive 32D cells did not change significantly during 24 hours of nocodazole treatment. These data indicate that Bcr-Abl—expressing cells undergo mitotic slippage, which is a typical response for cells with compromised mitotic checkpoint. As a mitotic slippage correlates with chromosome decondensation, to strengthen our previous observations, nuclear morphology was analyzed (Fig. 2C). After a gradual accumulation of cells with condensed chromosomes in all cell lines, the strong decrease of metaphase-arrested C2 and C4 cells and chromatin decondensation were observed (pointed by arrowheads). These changes did not correlate with the morphologic symptoms of cell death. The amount of metaphase-arrested 32D cells only slightly decreased and condensed chromosomes, which were visible till the end of the treatment. Moreover, cells with mitotic catastrophe symptoms (indicated by arrow) were observed only in the 32D population. Altogether, we showed that Bcr-Abl—expressing cells are not permanently arrested in metaphase but may exit mitosis despite continuous nocodazole treatment. It suggests that Bcr-Abl—expressing cells possess a compromised SAC. Because cyclin B1 degradation is required for mitosis exit, we examined its level to confirm our previous observations (Fig. 2D, left). In 32D cells, a high level of cyclin B1 persisted beyond 18 hours of treatment. C2 cells accumulated cyclin B1 during the first 12 hours, but later the protein level decreased significantly. For C4 cells, the drop was even more rapid, and already after 18 hours, cyclin B1 was undetectable. Typically, the signaling on mitotic slippage involves the cyclin B1 degradation, which correlates with the decrease of cyclin B1—dependent Cdk1 activity. Thus, we did Cdk1 immunoprecipitation followed by Cdk1 kinase assay in untreated or nocodazole-treated cells. Data presented Cdk1 activity in 32D, C2, and C4 cells (Fig. 2D, right). As expected, the Cdk1 activity in 32D cells increased on 6 hours of treatment with nocodazole, as a result of the accumulation of cells in mitosis. At later time points, the slight, however, not significant decrease of Cdk1 activity was detected, which correlated with the level of cyclin B1 detected in these cells. Unexpectedly, we found the upregulation of the Cdk1 activity in C2 and C4 cells undergoing mitotic slippage, apart from the cyclin B1 degradation. These surprising results are consistent with the noncanonical, cyclin B1—independent Cdk1 activity found in cells after mitotic slippage and described recently by Mantel et al. (16). They show that cells adapted to the SAC dysfunctions undergo mitotic slippage and maintain elevated levels of Cdk1 in an activated-like state whereas mitotic cyclin B1 is undetectable. Taken together, the disappearance of cyclin B1 and the decreased level of MPM-2 after 18 hours of nocodazole treatment, together with arrest with decondensed chromatin and 4C DNA

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**Figure 3.** Cells expressing Bcr-Abl undergo mitotic slippage in response to spindle checkpoint activation. A, cell cycle analysis done by the pulse BrdUrd incorporation assay. Cells were incubated with nocodazole for 24 h, and BrdUrd was added for 20 min. Cells were fixed, immunostained, and analyzed by flow cytometry. The percentage of BrdUrd-positive cells indicating cells in the S phase was calculated. Data are presented as mean ± SEM from three independent experiments. ****, P < 0.0005, versus control cells, by Student’s t test. B, expression profiles of p53 and p21 proteins estimated by Western blotting. Data in all panels are representative of three independent experiments. β-Actin as loading control. C, p21 levels in untreated and nocodazole-treated cells. Densitometry data from three independent experiments are shown as mean ± SEM.
content, indicate that Bcr-Abl-expressing cells possess compromised mitotic checkpoint and undergo transient mitotic arrest followed by mitotic slippage on nocodazole treatment; however, the role of Cdk1 kinase in this process remains unclear.

**Cells with a high level of Bcr-Abl undergo polyploidization on nocodazole treatment.** The general role of the mitotic slippage is to protect cells from entry to the next S phase of cell cycle. To confirm that after a 24-hour exposure to nocodazole Bcr-Abl-expressing cells are indeed arrested at a postmitotic checkpoint and do not enter S phase, we did the cell cycle analysis using pulse labeling with BrdUrd followed by flow cytometry analysis (Fig. 3A). We found that, after 24 hours of nocodazole treatment, 12.6% of 32D cells, 7.2% of C2, and 3.8% of C4 cells were BrdUrd positive, indicating cells in S phase. In contrast, 40% to 50% of control cells were found to be positive after 20 minutes of pulse labeling. Thus, at least at 24 hours, very little of nocodazole-treated cells were found in the S phase. Cells undergoing mitotic slippage are protected against polyploidy by activation of the postmitotic checkpoint, which depends on p53 and p21 activation (17). To further investigate the postmitotic checkpoint function, we checked the expression of these two proteins in nocodazole-treated cells (Fig. 3B). In 32D cells, a strong induction of p53 was observed, with only a transient increase in p21 level. These data, together with the viability study, indicated the p53-dependent death of 32D cells. In C2 and C4 cells, the levels of both p53 and p21 were increased. A 4-fold increase of p21 was observed in C2 cells and persisted until 48 hours. In C4 cells, the increase was much slower and reached only 2-fold at that time (Fig. 3C).

Because our results strongly indicated that a high Bcr-Abl level might have influenced the postmitotic checkpoint and are not fully protected against polyploidy, we did DNA content analysis of cells subjected to prolonged nocodazole treatment (Fig. 4A). 32D cells did not undergo polyploidization. In the C4 population, we detected 12% of polyploid cells, whereas in the C2 population, the percentage of polyploid cells did not exceed 4%. To check whether polyploid cells are able to enter mitosis, which could lead to aneuploidy, we did an analysis of the mitotic index by MPM-2 staining together with DNA content analysis after 72 hours of incubation with nocodazole. MPM-2-positive population (mitotic cells) were delineated as R1 region (Fig. 4B). Region R2 shows cells positive for MPM-2 with more than 4N DNA content, indicating that polyploid cells were able to reenter a next round of mitosis (Fig. 4B). In that subpopulation, we found >5% of nocodazole treated, high Bcr-Abl-expressing cells. These observations show that cells expressing Bcr-Abl at a high level are not able to effectively activate the tetraploid checkpoint on prolonged nocodazole treatment. It leads to their polyploidization and aberrant cell division.

**Bcr-Abl–expressing cells show high levels of aberrant mitoses and centrosome multiplication.** Our results presented, thus far, showed that Bcr-Abl–expressing cells treated with nocodazole had a compromised mitotic checkpoint. We expected that this defect should be visible in untreated cells as well. Thus, we investigated the morphology of mitotic nuclei and possible aberrations (Fig. 5A and B). In untreated Bcr-Abl–expressing cells, especially in C4, we found a high frequency of defective mitotic spindles, lagging chromosomes, anaphase bridges, and multinucleated cells. Even when a bipolar spindle was formed, some chromosomes failed to align correctly at the metaphase plate and remained lagging or unattached. Seventeen percent of C2 and 31% of C4 cells undergoing mitosis contained defective mitotic nuclei. In the parental cells, such abnormalities occurred in <6% of nuclei. These data show that mitosis deregulation is an immanent feature of Bcr-Abl–expressing cells and its severity correlates with the level of Bcr-Abl expression. To verify whether Bcr-Abl activity is responsible for the mitosis perturbances, we did long-term culture in the presence of a low dose of imatinib (0.25 μmol/L). After 2 weeks, the amount of aberrant mitoses, calculated as described above, decreased to 7.2% in C2 and 3.8% in C4 cells (not shown). The level of mitotic alterations observed in imatinib-treated Bcr-Abl–expressing cells was similar...
to that found in parental 32D cells (∼6%). That data supported our statement that Bcr-Abl expression correlates with generation of aberrant mitoses.

Because deregulation of division may produce cells with supernumerary centrosomes, we evaluated the number of centrosomes in untreated cells (Fig. 5C). The majority of the cells contained the correct number of centrosomes (1 or 2), but we also found C2 and C4 cells, but no 32D ones, that had multiple centrosomes (3–5). These observations regarding nontreated cells support our previous findings showing defective mitosis control associated with Bcr-Abl expression in cells treated with nocodazole.

Mitotic spindle defects caused by Bcr-Abl result in resistance to microtubule poisons. Because our studies showed failure of the mitotic checkpoints caused by Bcr-Abl, we decided to examine the biological consequences of these perturbances. The resistance to microtubule poisons is a result of the SAC dysfunction. Thus, we decided to address the sensitivity to two agents, nocodazole and paclitaxel (Fig. 6A and B, respectively). We found that 32D cells were highly sensitive to both agents and only ∼20% of viable cells were detected after 48 hours of treatment. In contrast, Bcr-Abl-expressing cells were resistant to nocodazole and paclitaxel. The viability of C2 and C4 cells after 48 hours of treatment was between 60% and 80%. To investigate whether the decreased sensitivity is a result of Bcr-Abl expression, we treated cells with imatinib, combined with nocodazole or paclitaxel. To prevent cell death as a result of IL-3 dependence occurring on Bcr-Abl kinase inhibition, RPMI was supplemented with WEHI conditioned medium as a source of IL-3. Although on IL-3 supplementation no cell death after addition of imatinib alone was detected (not shown), imatinib sensitized Bcr-Abl-expressing cells to spindle poisons. We noted that, for C2 and C4 cells treated with imatinib together with nocodazole or paclitaxel, the fraction of dead cells increased, showing the same sensitivity as the parental cells. Together, our results show Bcr-Abl-mediated failure of mechanisms controlling mitosis and the biological significance of these dysfunctions regarding cell division, ploidy control, and sensitivity to antimitotic agents.

Discussion

Genomic and chromosomal instability has been suggested to participate in the progression of CML (6, 18–20). Defects in genome maintenance observed in cancer cells are usually a result of dysfunctions in cell cycle checkpoints and DNA repair. Here, we found that the Bcr-Abl impaired a SAC as well as a postmitotic checkpoint. Our data provide evidence that Bcr-Abl expression led to a substantial downregulation of the BRCA1 protein and decreased expression of the mitotic checkpoint components. Recent findings describe BRCA1 as one of the critical regulators of the spindle checkpoint in both mouse and human cells (9). In a model deficient for a full length of the Brca1 isoform, a number

![Figure 5. Bcr-Abl-expressing cells show high levels of aberrant mitoses and centrosome amplification. A, nuclear morphology of mitotic cells after DAPI staining (×400). Typical pictures presenting lagging chromosomes (a, e), anaphase bridges (c), monopolar (b), and multipolar (d, f) spindles, as well as multinuclei (g, h), are indicated by arrows. B, frequency of aberrant mitoses in 32D, C2, and C4 cells. Three hundred cells were counted in three separate experiments; all types of abnormalities were counted together. Columns, mean; bars, SEM. *, P < 0.05; **, P < 0.005, versus untreated, by Student t test. C, representative images showing centrosome number in untreated cells (×400). Cells were stained with anti-γ-tubulin antibody (green) and counterstained to detect DNA (red).]
of genes involved in the spindle checkpoint regulation were decreased. BRCA1 knockdown in human prostate and breast cancer cells caused not only downregulation of genes implicated in the SAC but also centrosome malfunctioning, leading to aberrant mitoses (8, 21). Our data showing downregulation of mitotic genes expression after BRCA1 depletion in 32D cells are in agreement with these reports. According to the literature, BRCA1 can participate in gene transcription by interacting with the RNA polymerase II holoenzyme complex, with multiple transcription factors, or by chromatin remodeling as a result of interaction with histone acetyltransferases (22). However, the interaction between BRCA1 and promoters of its target genes is still not clear and needs further studies. BRCA1 protein downregulation caused by Bcr-Abl has been already once reported (7). Authors concluded that the tyrosine kinase activity plays a role in the BRCA1 downregulation, which occurs after transcription. We also showed that imatinib treatment protects cells from BRCA1 as well as Mad2, Bub1, Bub3, and BubR1 decrease, confirming the role of Bcr-Abl activity in these processes. Our experiments with MG132 indicated that the difference is not a result of protein degradation, rather the level of protein synthesis.

We found that Bcr-Abl–expressing cells were unable to efficiently activate the mitotic checkpoint. Moreover, cells escaped mitosis and underwent mitotic slippage on nocodazole treatment. Importantly, the prevalence of those effects correlated with the level of Bcr-Abl, suggesting that the defectiveness of the mitotic control increases with the increasing Bcr-Abl expression. Our findings are in agreement with studies by other groups reporting downregulation of mitotic checkpoint components in cancer cell lines and patient samples (23). It was shown that BUBR1 and BUB3 haploinsufficiency or a stable partial downregulation of MAD2 was sufficient to inactivate the mitotic checkpoint (24, 25). This was associated with the generation of gross aneuploidy as a result of spindle dysfunction and loss of mitotic control.

Recent studies using knockout mice showed that a complete loss of the SAC function resulted in early embryonic lethality, whereas weakened activity promoted cancer development (26–28). This may explain why only partial but not complete downregulation of spindle checkpoint genes is observed in human cancers (29, 30). Indeed, we can conclude that the Bcr-Abl leads to checkpoint weakness rather than complete dysfunction. The permanence of metaphase arrest depends on the level of Bcr-Abl expression. It cannot be neglected that other data show overexpression of the mitotic spindle genes causing similar final effects (31, 32). It seems that any impairment of SAC leading to loss of mitosis control, if not lethal, will cause chromosomal instability.

It is widely accepted that mammalian cells enter the G1 phase with 4N DNA after slippage from prolonged drug-induced mitotic block. This 4NG1 arrest, called the post-mitotic checkpoint, prevents polyploidy (17, 33, 34). The
canonical response on mitotic slippage involves cyclin B1 degradation correlating with the decrease of cyclin B1-dependent Cdk1 activity. Contrary to this widely accepted mechanism, the noncanonical, cyclin B1–independent Cdk1 activity was found in cells after mitotic slippage and described recently by Mantel et al. (16). It is the first report of such cellular condition, which is called by authors an “intermediate 4N stage or mitosis crisis subphase” and is a consequence of aborted mitosis. Moreover, studying the response on a single-cell level, the authors show that cells adapted to SAC dysfunctions undergo mitotic slippage and maintain elevated levels of Cdk1 in an activated-like state whereas mitotic cyclin B1 is undetectable. The role of this unconventional Cdk1 activity and the possible mechanism leading to its activation are still unknown. The Cdk1 activation, apart from the degradation of cyclin B1, was also found by us in nocodazole-treated C2 and C4 cells, which escaped mitosis and underwent mitotic slippage. Although this finding was unexpected, it is consistent with the data presented by Mantel et al. (16). This finding is novel and still controversial; however, we cannot exclude that the noncanonical cyclin B1–independent Cdk1 activation is also present in Bcr-Abl–expressing cells. In our study, particularly in high Bcr-Abl–expressing cells, we observed an increased number of polyploid cells generated on prolonged nocodazole treatment (48–72 h). Polyploids were not detected at 24 hours, indicating that at this time point cells are still arrested at 4NG1 and do not enter S phase yet, which was confirmed by BrdUrd incorporation. Appearance of polyploid cells indicates a compromised postmitotic checkpoint. It correlated with significantly lower p21 level detected in nocodazole-treated C4 cells in comparison with C2 cells. According to literature, the polyploid cells can initiate cell death, remain in metabolically active state without further divisions, or continue to divide with an abnormal DNA content (35). We observed a low level of death during polyplloidization, indicating that high Bcr-Abl–expressing cells possess mechanisms to survive that state. The observation that Bcr-Abl–expressing polyploid cells were able to undergo a next round of aberrant mitosis has very important implications for the disease progression. Chromosomal instability in dividing polyploid cells may serve as a source of aneuploidy progeny cells. They may undergo random and spontaneous chromosome loss leading to heterogeneous populations. Our observations are consistent with the working mechanistic model of CML blast crisis (36). Bcr-Abl is responsible for progressive genomic instability, which occurs at the CML stem cell level and/or in later CML progenitor cells. Moreover, the degree of the genomic instability is proportional to the level of Bcr-Abl activity, as it acts as an amplifier of the unstable phenotype.

Finally, we showed that Bcr-Abl–expressing cells with a compromised SAC were more resistant to nocodazole or paclitaxel treatment than parental cells and reduced susceptibility correlated with the level of Bcr-Abl. The relationship between the mitotic checkpoint function and cellular sensitivity to several types of anticancer drugs has particular clinical relevance (23, 35). It has been shown that SAC defects decrease sensitivity not only to antimitotic drugs but also to DNA-damaging agents. Data presented here clearly show that Bcr-Abl expression affects the SAC and postmitotic checkpoint and suggest that inactivation of the spindle checkpoint may contribute to chromosomal instability. In addition, defective SAC confers a growth advantage enabling cells to tolerate aneuploidy and to escape apoptosis, which could have tremendous implications. We propose that the downregulation of BRCA1 by Bcr-Abl could be a mechanism responsible for the modulation of the SAC function.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Expression of Oncogenic Kinase Bcr-Abl Impairs Mitotic Checkpoint and Promotes Aberrant Divisions and Resistance to Microtubule-Targeting Agents

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