Potentiation of Chlorambucil Cytotoxicity in B-Cell Chronic Lymphocytic Leukemia by Inhibition of DNA-dependent Protein Kinase Activity Using Wortmannin

Garyfallia Christodouloupolou, Catherine Muller, Bernard Salles, Rehan Kazmi, and Lawrence Panasci

Abstract

In this study, we examined the ability of wortmannin to modulate chlorambucil (CLB) cytotoxicity in lymphocyte samples from patients with B-cell chronic lymphocytic leukemia (B-CLL). It has been suggested previously that enhanced cross-link repair is a primary mechanism of resistance to nitrogen mustards (NMs) in B-CLL. DNA-dependent protein kinase (DNA-PK) is involved in the repair of double-strand breaks and in rejoining steps in recombination mechanisms. Mutants defective in this process are hypersensitive to alkylating agents. We have recently demonstrated that the activity of DNA-PK is a determinant in the cellular response of B-CLL to CLB. The DNA-PK gene has homology to the PI10 phosphatidylinositol 3-kinase 3-kinase (PI3-K). Wortmannin, an inhibitor of PI10 PI3-K, also inhibits DNA-PK activity in vitro. We investigated the effect of wortmannin on DNA-PK activity and CLB toxicity in the lymphocytes from 11 patients with B-CLL. Our results demonstrate that DNA-PK activity is decreased after exposure to wortmannin in a dose-dependent manner. Wortmannin, at nontoxic concentrations, synergistically sensitized B-CLL lymphocytes to the effects of CLB. Moreover, we observed a significant correlation when we compared the fold decrease in DNA-PK activity and the synergistic value (I), obtained when wortmannin was used at 0.1 µM. In the resistant B-CLL lymphocyte samples, there was a highly significant correlation between the ability of wortmannin at 0.1 and 0.25 µM to decrease the level of DNA-PK activity and to increase CLB sensitivity. In a model of primary human tumor cells, our findings suggest that the inhibition of DNA-PK activity may be a powerful way to overcome resistance to NMs such as CLB and point to new possibilities to improve the effectiveness of NM therapy.

Introduction

The appearance of a resistant cell population on relapse of an originally responsive tumor is one of the major limiting factors in chemotherapy. Understanding the molecular aspects of tumor cell sensitivity or resistance to various chemotherapeutic agents is an important determinant in developing more effective treatment strategies. In an effort to develop a clinically relevant model to investigate the mechanisms of NM resistance, we have been studying lymphocytes from patients with B-CLL. B-CLL is a disease characterized by the proliferation of abnormal, developmentally regulated, immature B cells that accumulate in the peripheral blood of affected patients. Patients are commonly treated with single-agent NM therapy, usually CLB, for many years, which eventually results in the development of resistance. Alkylation of the DNA and, more importantly, the interstrand cross-linking of DNA is considered to be responsible for the toxicity of NMs (1). Our previous studies have demonstrated that lymphocytes from treated resistant patients have an enhanced capacity to remove cross-links compared with those of untreated patients (2). Furthermore, we have demonstrated that NM resistance in B-CLL correlates with resistance to other bifunctional alkylating agents but not UV light or methyl methane sulfonate (3). This pattern of resistance is consistent with an Escherichia coli model in which recombination repair is implicated (4).

We demonstrated recently that the activity of DNA-PK is a determinant in the B-CLL lymphocyte response to CLB and participates in the development of drug therapy-resistant disease (5). The increase in DNA-PK activity might contribute to the enhanced cross-link repair that we defined previously as a primary mechanism of resistance to NMs in B-CLL. DNA-PK consists of two components, the heterodimer Ku autoantigen and the catalytic subunit, DNA-PKcs (6, 7).

Ku binds to double-strand ends and other discontinuities in the DNA and recruits the catalytic subunit of the complex (7). The active DNA-PK complex then acquires the capacity, at least in vitro, to phosphorylate many DNA-bound proteins in the vicinity (8). The repair process involves rejoining of broken ends of DNA by a homology-independent mechanism (9). Given the high radiation sensitivity of mutants defective in this process, this appears to be the principal pathway for DSB repair (10). Because some of these mutants are also hypersensitive to alkylating agents, this pathway may also be involved in the repair of cross-links (10, 11). Subsequent phosphorylation of DNA-bound proteins by DNA-PK is thought to facilitate the processes of DNA repair, recombination, transcription, and replication.

Because DNA-PK activity is involved in the response of B-CLL to NMs, the use of DNA-PK inhibitors is likely to improve the cytotoxicity of CLB in these cells. DNA-PKcs belongs to the PI3-K family by virtue of the high sequence homology of its kinase domain with PI10 PI3-K, a key enzyme involved in transducing growth factor responses mediated by tyrosine kinase. A potent inhibitor of PI3-K is the fungal metabolite, wortmannin. Wortmannin is noncompetitive and irreversible in action, with an IC50 in vitro of 1–5 nm. It has been demonstrated recently that wortmannin sensitizes cells to ionizing radiation (12). Important data from a subsequent study implicated inhibition of DNA-PK and the consequent inhibition of DSB repair as the mechanism whereby wortmannin potentiates the cytotoxicity of ionizing radiation (13). We have therefore hypothesized that wortmannin may have the ability to potentiate CLB in B-CLL samples by inhibiting the activity of DNA-PK. The results obtained from 11 patients are presented here.

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2 To whom requests for reprints should be addressed, at Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis Jewish General Hospital, 3755 Côte Ste-Catherine, Montreal, Quebec, H3T 1E2 Canada. Phone: (514) 340-8260; Fax: (514) 340-7502; E-mail: gchrisl@po-box.mcgill.ca.
3 The abbreviations used are: NM, nitrogen mustard; B-CLL, B-cell chronic lymphocytic leukemia; CLB, chlorambucil; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; PI3-K, phosphatidylinositol 3-kinase; DBS, double-strand break; MTT, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.
Materials and Methods

Drugs. CLB and wortmannin were obtained as powder from Sigma Chemical Co. and were dissolved in DMSO at a concentration of 20 and 10 mM, respectively. Immediately before addition to the cells, CLB was diluted in PBS, and wortmannin was diluted in water such that the final concentration of DMSO never exceeded 0.5%.

B-CLL Lymphocyte Cell Culture and MTT Cytotoxicity Assay. All patients were diagnosed with B-CLL and separated into two groups according to the following clinical criteria: (a) untreated patients, the vast majority of whom will respond to one of the NMs if and when they are treated; and (b) treated resistant patients, those who were treated with CLB for at least 3 months and failed to have a 25–30% reduction in their peripheral lymphocyte count (14). In addition, when the lymphocytes were tested for in vitro resistance, the LDL50 of CLB in the lymphocytes from resistant patients was at least 6-fold greater than the average LDL50 of the lymphocytes from untreated patients. The B lymphocytes from 11 patients (6 untreated, 4 treated and 1 sample, R2, which was de novo resistant) were used. Lymphocytes were isolated from the peripheral blood of B-CLL patients by centrifugation as described previously (14). To study the synergistic effects of wortmannin and CLB, freshly isolated lymphocytes were incubated at 37°C in the presence of various nontoxic concentrations of wortmannin (0.1 μM–0.5 μM) for 1 h prior to the addition of CLB. The wortmannin was left in the culture throughout the 3-day incubation period. The MTT assay was performed as described previously (3).

Analysis of Synergy. Synergy was determined by the formula:

\[ \frac{a}{A} + \frac{b}{B} = 1 \]

where \( a \) is the concentration of CLB required to produce 50% kill of control values in combination with the inhibitor at concentration \( b; A \) is the concentration of CLB required to produce 50% kill of control values in the absence of inhibitor; and \( B \) is the concentration of inhibitor required to produce 50% kill of control values in the absence of chlorambucil. According to this formula, when \( I < 1 \) the interaction is synergistic, when \( I = 1 \), the interaction is additive, and when \( I > 1 \), there is an antagonistic interaction (15).

Cell Extracts. Whole-cell extracts were prepared as described previously with slight modifications (16). Briefly, cell pellets were quickly thawed and resuspended in extraction buffer (1 × 10^6 cells per 100 μl) containing 50 mM NaF, 20 mM HEPES (pH 7.8), 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, and 0.5 mM DTT in the presence of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 1.5 μg/ml pepstatin), then frozen in liquid nitrogen and thawed at 30°C three times. After centrifugation for 30 min at 4°C, supernatants were stored at −70°C. Protein concentrations were determined using the Bio-Rad protein assay.

DNA-PK Activity. The DNA-PK "pull-down" kinase assay was performed as described previously (16). In this assay, total cell extracts were incubated with double-stranded DNA-cellulose, and then unbound material was removed by repeated washing. Because DNA-PK is activated when bound to DNA-cellulose, the immobilized enzyme was assayed simply by adding p53 peptide and [γ-32P]ATP. Each sample was assayed in the presence of the peptide EPPLSQEAFADLLKK, part of the p53 protein that is phosphorylated by DNA-PK, and the mutated peptide EPPLSQEAFADLLK, as a negative control. DNA-PK activity is expressed as the difference of radioactivity incorporated by the substrate minus the mutated peptide for a given extract.

Statistical Analysis. The \( P \) and \( r \) values were obtained using an unpaired t test and a simple linear regression analysis, respectively. All computations were performed using StatView 512 V0.12.

Results

We had previously determined that DNA-PK activity, a target kinase for wortmannin in vitro (6), is a determinant in the cellular response of B-CLL lymphocytes to CLB and participates in the development of drug-therapy resistant disease (5, 6). Accordingly, in our series, the mean DNA-PK activity was found to be significantly higher in the resistant samples compared with the sensitive ones (7180.8 ± 1564.9 cpm versus 3989.8 ± 1308.7 cpm, respectively; \( P < 0.005 \)). Moreover, linear regression analysis showed a highly significant correlation between CLB LD50 and the level of DNA-PK activity (\( r = 0.818, P = 0.0021 \)) in these samples, confirming our recent data.

We next examined the effect of wortmannin on DNA-PK activity present in CLL lymphocytes after exposure to increasing concentrations of this compound for 2 h. After this incubation period, cell extracts were prepared and assayed for DNA-PK activity as described previously (17). As shown in Fig. 1, extracts from wortmannin-treated CLL lymphocytes displayed a decrease in DNA-PK activity as the concentration increased. The observed decrease in DNA-PK activity was not due to a toxic effect of wortmannin, as assessed by the results of the MTT assay (Table 1).

To investigate the effect of wortmannin on the CLB chemosensitivity profile of CLL lymphocytes, we first determined whether wortmannin alone displayed significant cytotoxicity. Lymphocyte samples were exposed to a dose range of wortmannin of up to 20 μM, and the surviving fraction of the cells was determined using the MTT assay. This study allowed us to determine that wortmannin at concentrations ≤0.5 μM displayed minimal cytotoxicity in the majority of samples (see Table 1 for individual values). Interestingly, the wortmannin toxicity was not different between the CLB-resistant and -sensitive patients (Table 1).

We next examined the potential mechanism of action of wortmannin on CLB cytotoxicity. As shown in Table 1, \( I \) values of < 1 provide strong evidence that there is a synergistic interaction between these two drugs and suggest that wortmannin is able to modulate the activity of a key target involved in CLB toxicity to increase cell kill.

Wortmannin used at these concentrations was found to enhance CLB cytotoxicity in CLL lymphocytes sensitive to and resistant to CLB. This effect of wortmannin on CLB toxicity was dependent on the dose of wortmannin used (Table 2). It is interesting to note that in one sample, R3, wortmannin had a minimal inhibitory effect on CLB cytotoxicity in CLL lymphocytes sensitive to and resistant to CLB. This effect of wortmannin on CLB toxicity was dependent on the dose of wortmannin used (Table 2). It is interesting to note that in one sample, R3, wortmannin had a minimal inhibitory effect on CLB cytotoxicity in CLL lymphocytes sensitive to and resistant to CLB.
Materials and Methods. A synergistic interaction between chlorambucil and wortmannin was measured using the MTT assay. Cytoxicity profiles were also established for wortmannin. LD₅₀ represents the concentration of drug required to produce a 50% reduction in viable cells following a 3-day incubation. Toxicity refers to the cytotoxicity of the inhibitor alone (% cell death compared with controls). I is calculated according to the formula described in "Materials and Methods." I < 1 indicates a synergistic interaction between chlorambucil and wortmannin.

Concentration, wortmannin increased the CLB cytotoxicity by approximately 12-fold in the samples where this could be assessed.

To analyze the implication of DNA-PK in the effective potentiation of CLB toxicity observed in the presence of wortmannin, we then considered a relationship between its effect on CLB toxicity and DNA-PK activity. When we compared the fold decrease in DNA-PK activity with the synergistic value obtained, we found a statistically significant correlation that was most evident at a 0.1 μM concentration of wortmannin (r = 0.718, P = 0.0128). Similarly, a linear regression analysis demonstrated a highly significant correlation between the fold increase in sensitization to CLB and the fold decrease in DNA-PK activity in the resistant samples (r = 0.989, P = 0.0014 and r = 0.976, P = 0.0043 for 0.1 and 0.25 μM wortmannin, respectively).

These results, therefore, strongly suggest that wortmannin acts in a synergistic manner to sensitize B-CLL lymphocytes to CLB, possibly through the inhibition of DNA-PK activity.

Discussion

In this study, we examined the ability of wortmannin to potentiate CLB cytotoxicity and to inhibit DNA-PK activity. The data we present are consistent with a synergistic effect of wortmannin in enhancing CLB cytotoxicity, possibly via inhibition of DNA-PK activity and, as a consequence, inhibition of DNA repair in CLL lymphocyte samples.

Alkylating agents such as CLB covalently bind to DNA and induce the formation of various adducts, including interstrand cross-links. Because there is enhanced removal of cross-links in NM-resistant B-CLL cells (2), the inhibition of this repair process should be a powerful way to overcome resistance to alkylating agents such as CLB.

We have demonstrated previously that an increase in DNA-PK activity occurs during the development of NM therapy-resistant disease in B-CLL (5, 6). Thus, to the extent that enhanced DNA-PK activity does contribute to resistance, it should be possible to improve the efficacy of NMs against currently resistant tumors by inhibiting this activity. In this study, we demonstrate that wortmannin, a PI 3-K inhibitor that has previously been identified as an inhibitor of DNA-PK activity, at least in vitro (6), potentiates CLB toxicity in CLL lymphocytes either sensitive or resistant to CLB. In addition, our present data show, for the first time, that wortmannin may improve the effectiveness of NMs, especially in resistant samples, through its ability to inhibit DNA-PK activity. The dose range of wortmannin (0.1–0.5 μM) used to observe a potentiation of CLB toxicity is compatible with the dose range used to observe an effect on DNA-PK activity in vitro (6). Wortmannin has been shown to completely inhibit the ability of DNA-PK to phosphorylate the transcription factor Spl in cell extracts at a concentration of 500 nM (6). Another study elicits this effect by showing that wortmannin forms a covalent adduct in a conserved lysine residue in the kinase domain of DNA-PKcs, resulting in an irreversible inhibition of DNA-PK activity (17). Our findings suggest that the ability of wortmannin to inhibit DNA-PK activity may in fact be responsible for the increase in sensitization to CLB. This possibility is reinforced by the fact that inhibition of DNA-PK occurs at 100–500 nM, whereas the cellular activity of PI 3-K is completely abolished at wortmannin concentrations of <20 nM. The concentrations of wortmannin used in our study that result in chemosensitization are 5–25-fold higher than those needed to inhibit PI 3-kinase. Therefore, although we cannot exclude the possibility that wortmannin potentially targets PI 3-K, it is unlikely that inhibition of this enzyme plays a major role in potentiating CLB cytotoxicity in our lymphocyte samples.

In our study, we have demonstrated for the first time that DNA-PK activity is inhibited by wortmannin in lymphocytes. We also observed a correlation between the synergistic value obtained when CLB was used with 0.1 μM wortmannin and the fold decrease in DNA-PK activity.
activity that resulted with the same concentration of wortmannin. Our data also show that in the CLB-resistant lymphocytes, the increase in CLB sensitivity is correlated to the ability of wortmannin to inhibit DNA-PK activity. In addition, these results reinforce our previous conclusion that DNA-PK activity is implicated in the resistance phenotype to NMs (5, 6). It is interesting to note that sample R3, in which the synergistic interaction between CLB and wortmannin was not as pronounced as in the other samples, corresponded to the sample with the highest level of DNA-PK activity, even after incubation with wortmannin. This may suggest that the effect of wortmannin can only be observed below a threshold level of DNA-PK activity.

The mechanism of action of DNA-PK in regulating NM sensitivity remains to be elucidated; however, because the increased sensitivity to NMs has been observed in both Ku and DNA-PKcs mutant cell lines (10, 11), this indicates that the function of the entire DNA-PK protein complex (i.e., kinase activity) is involved in this process. The molecular features of DNA-PK suggest that DNA-PK is a direct participant in the machinery of repair of DNA cross-links. In accordance with the demonstrated role of DNA-PK in the repair of DSBs, a model of interstrand cross-link repair has been proposed that involves a dual excision on both DNA strands, leaving DSBs in DNA, which may be repaired by a DNA-PK dependent mechanism (10). Once bound to DNA ends, DNA-PK might phosphorylate and activate components of the DNA repair apparatus and thus restrict their activities to the appropriate cellular location (18). Another possibility is that DNA-PK initiates a signaling pathway to alert the cell to the fact that it has sustained damage (19).

We have demonstrated that wortmannin improves the effectiveness of NM cytotoxicity in CLL by overcoming intrinsic or acquired resistance due to its ability to inhibit DNA-PK activity. Because there is strong evidence that DNA-PK is involved in the regulation of cellular response to NMs, at least in lymphoproliferative disorders, identification of more specific DNA-PK inhibitors represents an important issue and should result in improved cancer therapy.

References


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