Modulation of Mitogen-activated Protein Kinases and Phosphorylation of Bcl-2 by Vinblastine Represent Persistent Forms of Normal Fluctuations at G2-M

Meiyun Fan, Lihua Du, Albert A. Stone, Kathleen M. Gilbert, and Timothy C. Chambers

Departments of Biochemistry and Molecular Biology [M. F., L. D., A. A. S., T. C. C.] and Microbiology and Immunology [K. M. G.], University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

ABSTRACT

Microtubule inhibitors, widely used in cancer chemotherapy, induce G2-M arrest and apoptosis and have in common the ability to stimulate Raf-1/Bcl-2 phosphorylation and activate c-Jun NH2-terminal protein kinase (JNK). These signal transduction pathways are thought to be activated in response to microtubule damage to promote apoptosis. However, Bcl-2 phosphorylation has been reported to occur at G2-M in non-apoptotic cells, raising the possibility that this and perhaps other signaling pathways altered by microtubule inhibitors reflect perturbations of normal mitotic events. In this study, we sought to test this hypothesis. We show that Bcl-2 phosphorylation and JNK activation, as well as extracellular signal response kinase and p38 inactivation, occur not only in response to vinblastine but also as discrete transient events at G2-M phase in untreated synchronized KB-3 cells. Thus, modulation of these pathways is not a response to microtubule damage; rather they occur normally at G2-M, and it is the extent, duration, and/or irreversible nature of the signals that distinguish a preapoptotic cell from one destined to divide. These findings provide novel insight into the relationship between mitotic and apoptotic signaling and the mechanism of action of antimitotic drugs.

INTRODUCTION

The Vinca alkaloid vinblastine has broad antineoplastic activity and has played a major role in cancer chemotherapy since its introduction nearly 40 years ago. The drug is an integral component of treatment for testicular cancer and Hodgkin’s and non-Hodgkin’s lymphomas and is used in combination therapy for carcinomas of the breast, lung, bladder, and for several other cancers (reviewed in Ref. 1). Vinblastine binds to tubulin subunits and inhibits tubulin polymerization, thus disrupting spindle microtubule dynamics and leading to mitotic block. For vinblastine and other Vinca alkaloids, there is a strong correlation between the drug concentration required to inhibit cell proliferation and that required to cause metaphase arrest (2). In a wide range of cancer cells, sustained metaphase arrest by vinblastine and other microtubule inhibitors induces cell death by apoptosis (reviewed in Ref. 3). The mechanisms that trigger apoptosis after metaphase arrest are largely unknown. However, microtubule inhibitors activate specific signal transduction pathways that may promote apoptosis. A common feature of microtubule inhibitors, as first shown for paclitaxel (4), is to stimulate phosphorylation of Bcl-2 through a pathway that may involve Raf-1 (5). Phosphorylation of Bcl-2 appears to reduce binding to Bax and render a cell more susceptible to apoptosis (3, 4), p53 is also induced by microtubule inhibitors in some cell types (3). In addition, microtubule inhibitors activate the stress-activated JNK4 pathway that can promote apoptosis (6–8).

Because JNK has been implicated primarily in stress responses, JNK activation by microtubule inhibitors may represent an acute response to microtubule damage. The phosphorylation of Bcl-2 has also been described as an important step from microtubule damage to apoptosis (4, 5). Recently, it has been shown that Bcl-2 phosphorylation occurs normally at the G2-M phase of the cell cycle (9–11), and that a pathway involving JNK may be involved (11). These studies raise the possibility that signal transduction pathways altered by microtubule inhibitors are manifestations of normal mitotic events, rather than stress responses to microtubule damage. In this study, we set out to test and broaden this hypothesis, focusing on MAPK pathways that we showed previously are selectively altered in KB-3 cells in response to microtubule inhibition (7). Our findings show that JNK activation and Bcl-2 phosphorylation occur not only in response to vinblastine but as discrete events at the G2-M transition in untreated synchronized cells. Furthermore, ERK and p38, which are inactivated by vinblastine treatment of KB-3 cells (7), also show cyclic fluctuations and are both inactivated during G2-M. The results provide novel insight into the mechanism of action of microtubule inhibitors and the relationship between mitotic and apoptotic regulation.

MATERIALS AND METHODS

Materials. Antibodies to p38, ERK1/2, Raf-1, ATF-2, Bcl-2, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to c-Jun and Bcl-XL were from Transduction Laboratories (San Diego, CA); antibodies to cyclin B and cyclin A were from PharMingen (San Diego, CA); and antibody to PARP was from Calbiochem (La Jolla, CA). Phosphospecific polyclonal antibodies for p38, ERK, c-Jun, and ATF-2 were obtained from New England Biolabs (Beverly, MA). Vinblastine, thymidine, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Proliferation Assays. The KB-3 human carcinoma cell line was maintained in monolayer culture at 37°C and 5% CO2 in DMEM, supplemented with 10% FBS, 2 mM l-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Inhibition of cell proliferation by vinblastine was measured by the MTT assay (12). Cells (2000/well) were plated in 96-well dishes, and after 24 h, the medium was replaced at zero time with fresh medium containing either vinblastine (0.1–100 nM in 0.1% DMSO) or vehicle alone. After incubation with drug for intervals of 1–12 h, the medium was replaced with drug-free medium, and MTT assay was performed 96 h after zero time. Continuous vinblastine treatment for 96 h was also performed. All treatments were performed in triplicate, and viability was expressed as a percentage of the untreated controls. Growth rates were measured by seeding 2 × 103 cells/well in 6-well dishes, adding vehicle or vinblastine the next day, and at daily intervals determining the number of trypan blue-excluding intact cells. Both adherent and nonadherent cells were pooled.

Cell Synchronization and Flow Cytometry. Cells were synchronized by the double thymidine block method (13). Cells (105) in a 100-mm dish were incubated in medium containing 2 mM thymidine for 16 h, released into normal medium for 9 h, and then incubated for 16 h in medium containing 2 mM thymidine. For DNA analysis, cells were fixed in 70% ethanol, washed in PBS.
RESULTS

We wanted to determine whether signaling pathways characteristically activated by microtubule inhibitors, including Bcl-2 phosphorylation (5) and JNK activation (6–8), as well as ERK and p38 inactivation documented previously in KB-3 cells (7), result from microtubule damage or reflect events occurring normally at mitosis. These possibilities are distinct both conceptually and mechanistically, and their elucidation should shed light on the mechanism of cytotoxicity of microtubule inhibitors and on our understanding of the relationship between mitotic and apoptotic regulation. Our strategy was to examine these parameters in vinblastine-treated cells compared with synchronized cells.

We first determined whether a correlation existed between the concentration of vinblastine required to inhibit cell proliferation and induce apoptotic cell death and that required to alter MAPK signaling and induce Bcl-2 phosphorylation. To define the threshold for vinblastine-induced death, KB-3 cells were exposed to a range of vinblastine concentrations (0.1–100 nM), either for short incubation periods up to 12 h or continuously, and cell viability was determined by MTT assay at 96 h. As shown in Fig. 1, where the relevant portion of the results is presented, inhibition of cell proliferation by vinblastine was both dose and time dependent. Exposure to 1 nM vinblastine for periods up to 12 h was not growth inhibitory, whereas continuous exposure at this concentration was inhibitory. On the other hand, treatment with 30 nM vinblastine for as little as 1 h was almost completely growth inhibitory. A graded response was observed at 10 nM vinblastine; cell viability was progressively lost with increasing treatment times. The most striking difference is seen at 10 nM compared with 30 nM vinblastine for 1 h of drug exposure, where relative cell survival was 75 and 8%, respectively, indicating that these conditions juxtapose the lethal threshold for vinblastine.

These results were confirmed by measuring growth rates over 96 h (Fig. 2). Control cells proliferated with a doubling time of ~21 h. Cells treated with 10 nM vinblastine for 1 h exhibited altered morphology, including decreased adherence and membrane ruffling, but after removal of the drug, normal cell morphology returned (data not shown). Such cells exhibited a slightly reduced proliferation rate, with 75–80% of the control value after 4 days (Fig. 2), consistent with the results of Fig. 1. Cells treated continuously with 10 nM vinblastine or with 30 nM vinblastine for 1 h or continuously failed to proliferate (Fig. 2). DNA analysis by flow cytometry demonstrated that cells under these conditions arrested at G2-M phase and eventually exhibited features characteristic of apoptotic cell death, including PARP cleavage, DNA ladderung, and nuclear fragmentation (Fig. 3 and data not shown).

Next we examined the activities of MAPKs (JNK, ERK, and p38) and Bcl-2 and Bcl-XL phosphorylation, under parallel conditions. Cells were untreated or treated for 1 h or continuously with 10 or 30 nM vinblastine, as in Fig. 2. Cell extracts were prepared at 16 h, a time point established in pilot experiments as optimal for the parameters under investigation, and analysis was performed. The results are shown in Fig. 3, along with the corresponding 4-day survival percentages. In control cells, cyclin B was not detectable; JNK was essen-

Fig. 1. Inhibition of cell proliferation by vinblastine. KB-3 cells were treated with vinblastine at concentrations of 1, 3, 10, or 30 nM, either for periods of 1, 4, 8, or 12 h, followed by incubation in drug-free medium, or continuously (96 h), as indicated, and cell viability was determined after 96 h by MTT assay, as described in “Materials and Methods.” Values are the means of triplicate assays. Results are expressed relative to untreated controls. Essentially identical results were obtained in an independent experiment.

Fig. 2. Inhibition of cell proliferation by vinblastine. KB-3 cells were untreated or treated with vinblastine at 10 or 30 nM for 1 h, followed by incubation in drug-free medium, or in the continuous presence of drug, and the number of viable cells was determined at 24-h intervals, as described in “Materials and Methods.” Values are the mean of duplicate assays.
during the 1-h drug exposure, and a proportion of total Bcl-2 was phosphorylated. In contrast, under each condition where cells were destined to die, cyclin B was accumulated; JNK was activated, as shown by NH2-terminal phosphorylation of c-Jun and ATF-2; ERK was inactivated; Bcl-2 and Bcl-XL were both present mainly in phosphorylated forms; and PARP cleavage was observed (the extent of which increased at later times). We also examined p38 activity under the same conditions of Fig. 3 and found that the enzyme was present in a phosphorylated/activated state under “survival” conditions but was present in an inactive state under the three “death” conditions (data not shown). This is consistent with our previous results showing p38 inactivation by lethal concentrations of vinblastine in KB-3 cells (7).

The results indicate a strong association between the concentration of vinblastine required to cause mitotic arrest, inhibit cell proliferation, and induce apoptotic cell death and that required to alter MAPK signaling and induce phosphorylation of Bcl-2 and Bcl-XL. To answer the key question of whether these events, individually or collectively, represent stress responses to microtubule damage or reflect normal events at mitosis, we examined the same parameters in synchronized cells. KB-3 cells were arrested at the G1-S boundary by the double thymidine block method (Fig. 4). After release of the block, cells proceeded through a full synchronous cell cycle, with most cells in S phase at 4–6 h, in G2-M at 8–10 h, and returning to G1 at 12 h, as determined by flow cytometry (Fig. 4). Synchronicity and phase distribution were supported by evaluation of cyclin A and B expression (Fig. 4). Evaluation of MAPKs and Bcl-2/Bcl-XL phosphorylation in synchronized cells is shown in Fig. 5. To allow a more detailed analysis, samples were prepared at hourly intervals through G2-M phase. Equal protein loading was established by actin expression, which remained unchanged, and expression of cyclin B provided an independent marker for G2-M progression (Fig. 5). Several striking observations were made:

(a) NH2-terminal phosphorylation of endogenous c-Jun and ATF-2 was observed for a very discrete period at 9–11 h from release, corresponding to the late G2-M phase, decreasing to undetectable levels as cells re-entered G1. c-Jun protein expression showed some fluctuation but was actually present at lower levels during this period, and ATF-2 protein expression was constant throughout, indicating...
that phosphorylation was not limited by substrate availability. Although p38 can also phosphorylate JNK sites in the NH2 terminus of ATF-2 (15), p38 is unlikely to be responsible for ATF-2 phosphorylation at G2-M because the enzyme becomes inactivated at this phase (see below). These findings demonstrate JNK activation as a normal event at G2-M phase in KB-3 cells.

(b) Both ERK and p38 became inactivated, starting at late S/early G2, and continuing through G2-M into G1 phase. The inactivation profiles of these MAPKs appear to be coordinated. Activated p38 may be responsible for the relatively low level of NH2-terminal ATF-2 phosphorylation observed in cells prior to release of the block (zero time in Fig. 5).

(c) Bcl-2 became phosphorylated in a transient manner for a very discrete period at 9–11 h after release, in late G2-M phase. Interestingly, we did not observe cell cycle-related changes in the phosphorylation of Bcl-XL.

(d) Finally, we analyzed Raf-1 in synchronized cells, because microtubule inhibitors induce hyperphosphorylation of this kinase in a wide variety of cell lines (5), including KB-3 cells (7). Although the mobility of Raf-1 appeared to vary somewhat throughout the cycle, a doublet at 10 h postblock corresponding to late G2-M was evident, indicating the presence of differentially phosphorylated forms at this phase.

**DISCUSSION**

In this study, we showed a very strong correlation between the vinblastine concentration required to inhibit KB-3 cell proliferation and induce cell death and that required to alter MAPK (JNK, ERK, and p38) signaling and induce phosphorylation of Bcl-2 and Bcl-XL. This is an important finding because many previous studies have used very high concentrations of microtubule inhibitors (1 μM and above), and the present results show these alterations correlate with lethal but not necessarily extreme conditions. We also demonstrated that many of the changes induced by vinblastine, including JNK activation, ERK, and p38 inactivation, and Bcl-2 phosphorylation occur at the G2-M phase of the cell cycle. This observation in particular has important conceptual and mechanistic implications, because it shows that these pathways are not activated in response to microtubule damage, as perceived commonly. Instead, they reflect persistent forms of normal G2-M fluctuations and appear amplified in mitotically arrested cells.

Other studies have used synchronized HeLa (9) or colon carcinoma (10) cells to show that Bcl-2 phosphorylation occurs during normal mitotic progression, suggesting this event is not cell type specific. In one study (9), Bcl-2 phosphorylation was observed over a 6-h period, from 6 to 12 h after release of HeLa cells from a double thymidine block. Our findings differ somewhat in that we observed Bcl-2 phosphorylation as an abrupt and discrete event, occurring over a short period of <3 h at G2-M (Fig. 5). This may reflect the high degree of synchrony we were able to achieve in this system (Fig. 4). Recent evidence using dominant-negative constructs to components in the JNK signaling pathway has suggested that JNK is the kinase responsible for mitotic Bcl-2 phosphorylation (11). We have also shown recently, using antisense oligonucleotides directed toward JNK isoforms, that JNK is responsible for vinblastine-induced Bcl-2 phosphorylation in KB-3 cells (16). Our finding that endogenous phosphorylation of both c-Jun and ATF-2 at JNK-specific sites occurs at precisely the same time in the cell cycle as Bcl-2 phosphorylation (Fig. 5) supports a role for JNK in the mitotic phosphorylation of Bcl-2.

During the cell cycle, the phosphorylation of c-Jun, ATF-2, and Bcl-2 is rapidly reversed as cells re-enter G1, whereas vinblastine induces sustained phosphorylation of these proteins. It is also evident that the phosphorylation-induced mobility shift of Bcl-2 at G2-M involves only a proportion of the Bcl-2 molecules, whereas more highly phosphorylated Bcl-2 species are induced in response to vinblastine (Figs. 3 and 5). This difference in the extent and duration of substrate phosphorylation, leading to the accumulation of highly phosphorylated JNK substrates in mitotically arrested cells, may be a key factor in promoting apoptosis. Sustained phosphorylation may be attributable to prolonged kinase activity or the failure of a relevant phosphatase(s) to activate normally. Therefore, it will be important to identify the regulatory mechanisms controlling these critical enzymes. Of additional significance is our finding that Bcl-XL was phosphorylated in response to vinblastine but not apparently during the cell cycle (Fig. 5). This modification may therefore represent an apoptosis-specific event important for cell fate in response to microtubule damage.

What might be the role of JNK activation and phosphorylation of c-Jun, ATF-2, and Bcl-2 during mitosis? In many systems, JNK activation is a proapoptotic signal, a notion supported by the recent development of JNK1/JNK2-deficient mouse embryos (17). The pos-
sibility that JNK is responsible for Bcl-2 phosphorylation at G2-M strengthens the connection between JNK signaling and apoptosis as phosphorylation appears to inhibit the antiapoptotic ability of Bcl-2 (11). Thus, mitotic cells may be preparing for apoptosis as a means to more readily eliminate cells that divide abnormally. The threshold for cell death may diminish after successful completion of mitosis, perhaps attributable in part to JNK pathway inactivation. One prediction of this hypothesis is that mitotic cells would be more susceptible to apoptotic stimuli. In preliminary experiments, we have compared asynchronous KB-3 cells and cells at G2-M (10 h after double thymidine block) and found that the latter are significantly more sensitive to several apoptosis-inducing stimuli including anisomycin, staurosporine, and vinblastine. Understanding the regulatory mechanisms that control JNK activation and inactivation at G2-M and the functional consequences of substrate phosphorylation will be important to further appreciate the role of this signaling pathway in mitosis and apoptosis. Future work in this area should help to clarify the molecular relationship between these fundamental cellular processes and the signaling mechanisms involved in apoptotic cell death by antimitotic anticancer agents.

ACKNOWLEDGMENTS

We thank Drs. Rick Drake and Tom Kelly for critical review of the manuscript and helpful suggestions.

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