Loss of Cell Cycle Control Allows Selective Microtubule-active Drug-induced Bcl-2 Phosphorylation and Cytotoxicity in Autonomous Cancer Cells

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Abstract

Lack of selectivity in the killing of tumor and normal cells is a major obstacle in cancer therapy. By inhibiting normal but not autonomous cell growth, we exploited the differences in cell cycle regulation to achieve a selective protection of nonautonomous cells against paclitaxel and other microtubule-active drugs. Tubulin polymerization, a primary effect of paclitaxel, can be dissociated from Bcl-2 phosphorylation and cytotoxicity in HL-60 cells. Growth arrest prevented paclitaxel-induced Bcl-2 phosphorylation and apoptosis without affecting paclitaxel-induced tubulin polymerization. We abrogated the effects of paclitaxel on MCF-10A immortalized breast cells, while preserving its effects on MCF-7 cancer cells. Unlike MCF-7 cells, MCF-10A cells were arrested by epidermal growth factor withdrawal, precluding paclitaxel-induced Bcl-2 phosphorylation. Furthermore, the inhibition of the epidermal growth factor receptor kinase with low doses of AG1478 arrested growth of MCF-10A but not MCF-7 cells. Pretreatment with AG1478 did not affect paclitaxel-induced Bcl-2/Raf-1 phosphorylation in MCF-7 but abrogated such phosphorylation in MCF-10A. Exploitation of growth factor dependency may allow the protection of normal cells from microtubule-active drugs.

Introduction

Microtubule-active drugs, such as taxanes and Vinca alkaloids, are important agents in cancer treatment (1). Although microtubule-active drugs have the capacity to kill most cancer cells, toxicity to normal cells limits their clinical use. Microtubule-active drugs cause microtubule dysfunction leading to mitotic arrest (2, 3). Mitotic arrest is accompanied by serine phosphorylation of multiple regulatory proteins, including Bcl-2, and precedes cell death: either apoptotic or nonapoptotic (4). In principle, a cell arrested in interphase by a nontoxic agent cannot enter mitosis and therefore cannot undergo mitotic arrest when exposed to a microtubule-active drug. Hence, arresting normal cells in interphase before treatment with microtubule-active drugs could increase the selectivity of microtubule-active drugs against cancer cells by minimizing undesired toxicity to normal cells.

The pioneering works by Pardee (5) and Pardee and James (6) proposed that loss of cell cycle control allows selective killing of transformed cells by chemotheraphy. Activation of oncogenes and inactivation of tumor suppressor genes result in autonomous growth of cancer cells (7). Also, aggressive cancer cells may lose sensitivity to growth inhibitors such as p21 (8). By definition, autonomous growth occurs independent of specific mitogens and signal transduction pathways; and interference with these pathways may be inconsequential to a cell with autonomous growth. It is thus conceivable that under specific conditions, growth arrest can be achieved in cells that have intact signal transduction pathways but not in dysregulated cancer cells. Here we demonstrate that selective growth arrest of

nonautonomous cells can abrogate the cytotoxic effects that are otherwise caused by PTX.2

Materials and Methods

Cell Lines. HL-60, a human myeloid leukemia cells, and MCF-7, a breast cancer cell line, were obtained from American Type Culture Collection and cultured in RPMI plus 10% fetal bovine serum. MCF-10A immortalized human breast epithelial cells were provided by Dr. David Salomon (National Cancer Institute, NIH, Bethesda, MD) and were cultured in DMEM-Ham’s F12 (1:1) medium containing 5% horse serum plus 20 μg/mL HEPES, 4 mg/ml insulin, 500 ng/ml hydrocortisone, and 20 ng/ml EGF (all from Collaborative Biomedical Products, Bedford, MA).

Reagents. PTX (Taxol) was a Bristol-Myers product; Vincristine and vincristine were Eli Lilly products (Indianapolis, IN). Epothilones A and B were provided by Dr. F. Lee (Bristol-Myers, Princeton, NJ) and dissolved in water. Phorbol 12-myristate 13-acetate (PMA/TPA; Sigma) and PD98059, a MEK inhibitor (New England BioLabs), were dissolved in DMSO at concentrations of 1 mm and 20 μm, respectively. AG1478, [4-(3-chloroanilino)-6,7-dimethoxyquinazoline] (Calbiochem), a potent and selective inhibitor of EGFR receptor (9), was solubilized as a 10 mm stock solution in DMSO.

Immunoblot Analysis. Proteins were harvested and were resolved on 12.5% SDS PAGE for p21WAF1/CIP1, Bcl-2, or β-tubulin, and on 7.5% SDSPAGE for Raf-1, PARP, and tubulin as previously described (10). If indicated, proteins were resolved on NuPAGE 4–12% Bis-Tris gel with 4-morpholinepropanesulfonic acid running buffer (NOVEX, San Diego, CA) according to the manufacturer’s instructions. Immunoblot was performed using the following antibodies: antihuman mononuclear WAF1 (Calbiochem), anti-Raf-1 rabbit polyclonal (C12, Santa Cruz, CA), antihuman Bcl-2 monoclonal (DAKO), anti-PARP rabbit polyclonal (Upstate Biotechnology, Lake Placid, NY), anti-Raf-1 rabbit polyclonal (C12, Santa Cruz), and antitubulin monoclonal antibodies (Sigma, St. Louis, MO).

MTT Assay. MTT assay was performed as described previously (10). In brief, cells were plated in 96-well flat-bottomed plates in 100 μl of medium and were exposed to the pharmacological agents on the following day. Two days after the last drug addition, 20 μl of 5 mg/ml MTT solution in PBS was added to each well for 4 h. The medium was discarded, and 170 μl of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a plate reader. Triplicate wells were assayed for each condition, and SDs were determined.

Mitotic Index. Cells were centrifuged, fixed, and stained with 4’,6-diamidino-2-phenylindole. For each sample, 100–500 cells were randomly counted by microscopy, and mitotic cells were scored by their lack of nuclear membrane and evidence of chromosome condensation.

DNA Synthesis. DNA synthesis was monitored by [3H]thymidine incorporation. In brief, HL-60 cells were plated in 96-well flat-bottomed plates, and MCF-7 and MCF-10A cells were plated in 24-well plates. The next day, cells were treated with drugs. After 16 h, cells were incubated with 1 μCi of [methyl3H]thymidine (Amersham) for an additional 4–8 h and then acid-counted by microscopy, and mitotic cells were scored by their lack of nuclear membrane and evidence of chromosome condensation.

Cell Cycle Analysis. Cells were harvested by trypsinization, washed with PBS, resuspended in 75% ethanol in PBS, and kept at 4°C for at least 30 min.
Before analysis, cells were washed again with PBS and resuspended and incubated for 30 min in propidium iodide staining solution containing 0.05 mg/ml propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton-X-100, and 1 mg/ml RNase A in PBS. The suspension was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACScan.

Results

Bcl-2 Phosphorylation, a Marker of Mitotic Arrest, Can Be Dissociated from Tubulin Effects. When microtubule-active drugs cause mitotic arrest of adherent cells, such as MCF-7, the mitotic cells become round and detached from the flask. We separated mitotic cells (detached) from interphase cells (attached) by shake-off. Immunoblot analysis of these two populations revealed a decrease in soluble tubulin, reflecting PTX-driven tubulin polymerization, in both cell populations (Fig. 1, attached and detached cells). The reduction in soluble tubulin was more pronounced in the interphase cells (attached) but also was observed in the mitotic cells, whereas Bcl-2 phosphorylation occurred exclusively in mitotic cells (Fig. 1).

Although Bcl-2 is an antiapoptotic protein (11), PTX-induced Bcl-2 phosphorylation leads to Bcl-2 inactivation (12, 13). It has been proposed that phosphorylation of Bcl-2 is a suicidal response to a mitotic arrest that otherwise might lead to abnormal chromosome segregation (14, 15). Mitotic arrest-associated Bcl-2 phosphorylation (16) precedes cell death, although cell death is not necessarily apoptotic. Apoptosis, a rapid cell death associated with caspase activation, was observed in HL-60 cells in response to PTX. In these cells, a Mr 22,000 fragment of Bcl-2 cleavage, one marker of caspase activation, was apparent (Fig. 1). In contrast, Bcl-2 cleavage was absent in MCF-7 cells that lack caspase-3. Slow cell death without caspase activation is typical for MCF-7 cells as well as for many other cancer cell lines. Regardless of the mode of death, Bcl-2 phosphorylation is a common early marker of PTX cytotoxicity in breast and leukemia cell lines.

PMA-induced Growth Arrest Abrogates PTX-induced Cytotoxicity in PMA-sensitive Cells. PTX and other microtubule-active drugs induce apoptosis in leukemia cells after 16–24 h of exposure (10, 17). It has been demonstrated that phorbol ester protects cells against chemotherapy (18, 19). As expected, in HL-60 cells, PTX decreased the amount of soluble β-tubulin (a marker of PTX-tubulin interaction), caused Bcl-2 phosphorylation, and resulted in PARP cleavage (a marker of apoptosis; Fig. 2A). PTX also inhibited DNA synthesis (Fig. 2B) and resulted in cell death (Fig. 2C). Phorbol ester (PMA) caused a dramatic p21 induction (Fig. 2C) and consequent growth arrest with an almost 100-fold inhibition of DNA synthesis (Fig. 2B) without causing cell death (Fig. 2C). In PMA-arrested cells (pretreated with PMA for 8 h), PTX still decreased the amount of soluble tubulin but did not cause Bcl-2 phosphorylation. Consistently with this, PMA pretreatment abrogated the cytotoxic effects of PTX, 

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**Fig. 1.** Tubulin alterations versus Bcl-2 phosphorylation. MCF-7 cells and HL-60 cells were incubated with 100 ng/ml PTX overnight, and an immunoblot for soluble β-tubulin and Bcl-2 was performed as described in “Materials and Methods” (see Ref. 12). D, detached (mitotic) cells; A, attached (interphase) cells. N/S, nonspecific band demonstrating equal loading in MCF-7 D and A cells. Lowest panel, the Mr 22,000 fragment of Bcl-2 detectable on a grossly overexposed blot. kDa, molecular weight in thousands.

**Fig. 2.** PMA-induced growth arrest abrogates PTX-induced cytotoxicity. HL-60 cells were incubated with 100 ng/ml PMA for 8 h or left untreated. Then untreated and PMA-pretreated cells were exposed to 100 ng/ml PTX, as indicated on the top of the figure. A, after 16 h of PTX treatment, cells were lysed and immunoblots for PARP, tubulin, Bcl-2, and p21 were performed as described in “Materials and Methods.” B, [3H]thymidine incorporation was performed after 2 days of PTX exposure. C, MTT assay was performed after 2 days of PTX exposure. D, growth arrest caused by PD98059 diminished PTX-induced cytotoxicity. HL-60 cells were treated with 60 μM PD98059 simultaneously with or before PTX (16 h before treatment). MTT assay was performed after 3 days as described in “Materials and Methods.”
thereby preventing PARP cleavage (Fig. 2A) and cell death (Fig. 2C). Pretreatment with PMA also protected HL-60 cells from cytotoxicity following other microtubule-active drugs, including the tubulin polymerizing agents epothilones A and B, and the depolymerizing drug, vincristine (data not shown). The cytoprotective effects of PMA were most pronounced when cells were pretreated for at least 8–12 h to allow for interphase arrest, thereby preventing PTX-induced mitotic arrest.

**Pharmacological Interruption of the MAPK Pathway Protects Growth Factor-dependent Cells.** As previously demonstrated (10, 20), MEK, an intermediate kinase in the MAPK pathway, is dispensable for the cytotoxicity of microtubule-active drugs. However, in growth factor-dependent cell lines, the MAPK pathway may be important for growth factor signaling and proliferation. Sixty μM PD98059, an inhibitor of MEK, inhibited proliferation of HL-60 cells, and when HL-60 cells were pretreated with the MEK inhibitor for 16 h, the cytotoxicity of PTX was reduced (Fig. 2D). However, when PD98059 was added simultaneously with PTX, it exerted no protection (Fig. 2D). Although it has been reported that PD98059 can protect cells regardless of delay of cell cycle progression (21), other studies did not support an involvement of MEK in PTX toxicity (10, 20). The fact that cyostatic doses of both an activator and an inhibitor of the MAPK pathway can provide cytoprotection emphasizes the role of cell cycle arrest in their activities.

**Selective Ablation of PTX-induced Bcl-2 Phosphorylation in MCF-10A but not MCF-7 Cells.** Withdrawal of EGF from MCF-10A cells, an immortalized mammary cell line, resulted in growth arrest in the G2/M phase (Fig. 3A, control versus EGF withdrawal). Stimulation of EGF-deprived cells with exogenous EGF synchronizes cells to enter the G2/M phase (Fig. 3A, +EGF). Treatment with AG1478, a selective inhibitor of the EGF receptor, abrogated the EGF-induced cell cycle progression in MCF-10A cells (Fig. 3A).

When a 6-h treatment with PTX was performed immediately after a 6-h treatment with EGF, neither Bcl-2 nor Raf-1 phosphorylation was detected (Fig. 3B). The absence of phosphorylation was consistent with cells remaining in the G1 phase (Fig. 3C). In contrast, when PTX was added after 16 h of EGF treatment, we observed Bcl-2 and Raf-1 phosphorylation, as evidenced by the slower migrating bands (Fig. 3B). Under these conditions, many cells had already reached the G2/M phases by the time of PTX exposure (Fig. 3C). These studies demonstrate that PTX can cause Bcl-2 and Raf-1 phosphorylation in EGF-stimulated cells, only if cells are allowed to reach mitosis. Thus, progression to mitosis is required for Raf-1/Bcl-2 phosphorylation.

We next compared MCF-10A cells, which, as was shown, are EGF-responsive with MCF-7, an EGF-independent metastatic cancer cell line. In agreement with EGF dependency, the growth of MCF-10A cells could be inhibited by low concentrations of AG1478, a selective inhibitor of the EGF receptor (Fig. 4A). Much higher concentrations of AG1478 were required to inhibit growth of MCF-7 cells (Fig. 4A), possibly by non-EGF receptor targets. By using low doses of AG1478, we could selectively arrest MCF-10 cells without arresting the autonomous MCF-7 cells. Thus, we exploited this difference to selectively manipulate the effects of PTX in MCF-10A versus MCF-7 cells. PTX caused Raf-1 and Bcl-2 phosphorylation when both cell lines were normally proliferating (Fig. 4B). Inhibition of EGF receptor kinase with 0.25 μM AG1478 caused marked growth arrest of MCF-10, with only marginal inhibition of MCF-7 cells (4 C). Pretreatment with AG1478 prevented Raf-1 and Bcl-2 phosphorylation in MCF-10A; in contrast, Raf-1 and Bcl-2 phosphorylation were observed in MCF-7 cells.

**Discussion**

Chemotherapeutic agents, including microtubule-active drugs, discriminate poorly between normal and cancer cells. We envisage a protection of normal cells that is based on four principles: (a) cancer cells are less dependent on growth factors and/or resistant to growth inhibitors; (b) it is possible to selectively arrest normal cells in the interphase; (c) microtubule-active drugs are cytotoxic because of their ability to induce mitotic arrest in cycling cells; and (d) cells arrested in the interphase may be insensitive to microtubule-active drugs. Here we have shown that cells with intact cell cycle control can be selectively protected from microtubule-active drugs. Microtubule-active drugs such as PTX are effective therapeutically, and substantial evidence has been accumulated that the ability of these drugs to kill cells is a result of mitotic arrest (4), although subsequent pathways leading to cell death are not fully elucidated. Phosphorylation of regulatory proteins, including Bcl-2, is associated with mitotic arrest and cytotoxicity. The events that are associated with Bcl-2 phosphorylation occur after tubulin dysfunction (upstream effects) and before cell death (downstream events). There are two reasons for disrupting the PTX effects at this level: (a) we do not fully understand the pathways from mitotic arrest to cell death to manipulate them in a cell type-specific manner; (b) autonomous growth, the most fundamental difference between normal and malignant cells, allows the selective arrest of the growth of normal but not autonomous cells. Thus, the
EGF-dependent immortalized breast cells underwent G0/G1 growth arrest after EGF withdrawal. This precluded Bcl-2 phosphorylation in the arrested cells. In contrast, MCF-7 cells were not affected by withdrawal of EGF. Although EGF withdrawal is not available as a therapeutic intervention, the dependence on EGF can be exploited using AG1478, a selective inhibitor of the EGF receptor kinase (Fig. 4). This allowed the arrest of MCF-10A with low doses of AG1478. As a result, AG1478 abrogated PTX effects in MCF-10A cells but not in MCF-7 cells. Although the toxicity toward breast tissue does not represent a dose-limiting toxicity for taxanes, using two cell lines with well-defined differences, such as dependence on the EGF signaling, shows that even such related cell lines could be selectively affected. Normal bone marrow and epithelial cells are particularly vulnerable to chemotherapy. Reversible and selective inhibitors of the cycle of epithelial and hematopoietic cells will be especially valuable.

Similarly, the autocrine growth factor-dependent HL-60 cells could be effectively arrested by PMA. This prevented Bcl-2 phosphorylation, caspase activation, and cell death that was otherwise caused by PTX. In addition, p21 may affect apoptosis through a cell cycle-dependent mechanism (22). PD98059, an inhibitor of MEK, did not interfere with PTX-induced cytotoxicity because MEK is not essential for PTX-induced apoptosis (10, 20). However, PD98059 inhibited growth of HL-60 cells. Therefore, when PD98059 was added 16 h before PTX, it increased resistance to PTX (Fig. 2), indicating that cytoreduction depends on the growth arrest. Certainly, most cells, normal or malignant and even the most autonomous, can be arrested by a wide variety of cytotoxic drugs that do not discriminate between normally and autonomously growing cells. Previously, we and others demonstrated that a treatment with some drugs abrogated PTX-induced Bcl-2 phosphorylation even in autonomous cancer cells (14, 23). The pretreatment of cancer cells with cytostatic agents has often been shown to be antagonistic with PTX (24). In addition to autonomous growth, other targets can be exploited for cytoprotection against microtubule-active drugs. As we previously found, cytoprotection can be based on a p35/p21-checkpoint, which allows one to discriminate normal and p35/p21-defective cancer cells by pretreatment with low cytostatic doses of DNA-damaging drugs (25).

In sequence-dependent clinical studies, less severe hematological toxicity has been reported in courses in which cyclophosphamide, a nonselective cytotoxic, was administered first and PTX was administered second (26). Although many questions should be answered before clinical recommendations are given, these approaches could potentially increase selectivity in killing of malignant cells while decreasing the side effects of chemotherapy.

References

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