Interleukin 6 Differentially Potentiates the Antitumor Effects of Taxol and Vinblastine in U266 Human Myeloma Cells

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ABSTRACT

Newer therapeutic strategies for the treatment of multiple myeloma have focused on antagonizing the growth-promoting functions of interleukin 6 (IL-6). In this study, we examined the antitumor effects of two mechanistically different microtubule poisons, Taxol and vinblastine, in U266 human myeloma cells and determined whether IL-6 altered these effects. Taxol and vinblastine led to a dose-dependent inhibition of [3H]thymidine incorporation and altered the DNA distribution pattern of U266 cells. Both drugs led to an increase in the proportion of cells in the sub-G1 fraction (<2N DNA). However, at the IC50 concentration, vinblastine, but not Taxol, increased the percentage of cells in the G2-M phase of the cell cycle. In the presence of IL-6, the DNA distribution pattern induced by Taxol or vinblastine was altered. Whereas IL-6 augmented the sub-G1 fraction and G2-M phase for Taxol-treated cells, only the G2-M phase was increased for vinblastine-treated cells. Furthermore, IL-6 enhanced the cytotoxicity of both drugs, which became evident only during recovery in cytokine-free and drug-free medium. However, the cytotoxicity of Taxol was augmented to a significantly greater extent than that of vinblastine (P < 0.001). Immunostaining with antibodies to α-tubulin and mitogen-activated protein kinase revealed co-localization of these two proteins within microtubule asters. In the presence of IL-6, the number of cells containing microtubule asters increased for Taxol treatment, but not for vinblastine treatment. These data indicate that IL-6 leads to differential modulation of the cytotoxicity of Taxol and vinblastine in U266 cells. Whereas recruitment of cells in the S phase of the cell cycle represents a major mechanism by which IL-6 potentiates the cytotoxicity of vinblastine, augmentation of the cytotoxicity of Taxol involves additional mechanisms. Furthermore, our data suggest that the microtubule-associated form of mitogen-activated protein kinase may play a role in IL-6-mediated enhancement of the cytotoxicity of Taxol. The clinical implications of these findings are discussed.

INTRODUCTION

Multiple myeloma is a B-cell neoplasia characterized by clonal expansion of malignant plasma cells in the bone marrow. Proliferation of these neoplastic cells is facilitated within a microenvironment of stromal and accessory cells, which provide the necessary cell-to-cell interactions as well as soluble growth-promoting mediators. Among the various mediators, IL-6 has emerged as a major growth factor regulating the proliferation of myeloma cells in vivo and in vitro (1–6). High levels of serum IL-6 have often been shown to correlate with disease progression (7, 8). Thus, therapeutic strategies that interfere with the production or signaling of IL-6 have been designed for the treatment of multiple myeloma. For example, antibodies to IL-6 (9), all-trans-retinoic acid (10–13), IFN-α and -γ (13–16), dexamethasone (12, 13, 17), and various combinations of these have proved effective in in vitro and some in vivo studies. In spite of these advances, the prognosis for myeloma is poor, and conventional chemotherapy prolongs survival by only a few years.

Taxol and the Vinca alkaloid vinblastine are two important cell cycle-dependent antitumor drugs. Unlike Vinca alkaloids, which are used for the treatment of hematological malignancies (18), Taxol is widely used for the treatment of solid tumors, especially breast and ovarian cancers (19, 20). The effect of Taxol in hematological malignancies has not been fully explored. Although the cytotoxic effects of both Taxol and vinblastine are thought to involve the arrest of cells in the G2-M phase of the cell cycle, the mechanism by which this is achieved differs. Taxol leads to irreversible polymerization of tubulin and stabilization of microtubules (21–23), whereas vinblastine binds to tubulin with high affinity and prevents microtubule assembly (24).

Recent studies on the molecular mechanisms by which Taxol leads to cell cycle arrest and cell death indicate that the drug modulates the activity and transcription of several key elements in signaling pathways governing cell cycle regulation and apoptosis. It has been reported that Taxol leads to activation of MAPK, also referred to as erk (25), c-raf-1 (26, 27), and cdc-2 kinase (28); to induction of p21WAF1 and p53 proteins (26); and to phosphorylation of bcl-2 (27, 29). In contrast, the molecular mechanisms involved in the cytotoxic effects of vinblastine are poorly understood.

In this study, we evaluated the cytotoxic effects of the two microtubule poisons, Taxol and vinblastine, in U266 human myeloma cells. Because the growth of this tumor is stimulated...
by IL-6, we determined whether IL-6 would alter the antitumor effects of these drugs. Our results indicate that the antiproliferative effects of Taxol and vinblastine are augmented by IL-6. However, the mechanism of action and degree of potentiation vary for these two drugs. Although IL-6 enhances the proportion of cells in the G2-M phase of the cell cycle for both Taxol and vinblastine treatments, it selectively potentiates a Taxol-induced increase in the sub-G1 (apoptotic) fraction and a sequestration of MAPK within microtubule asters.

MATERIALS AND METHODS

Cell Culture. The human myeloma cell line U266 was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented with l-glutamine and 15% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Reagents and Antibodies. Recombinant human IL-6 was a gift from Sandoz (East Hanover, NJ). Taxol was purchased from Sigma Chemical Co. (St. Louis, MO). Vinblastine sulfate for injection was obtained from Faulding Pharmaceuticals (Elizabeth, NJ). The following antibodies were used: (a) mouse antihuman α-tubulin (Sigma Chemical Co.); (b) rabbit antihuman erk-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (c) rhodamine-conjugated goat antimouse immunoglobulin (Sigma Chemical Co.); and (d) FITC-conjugated antirabbit immunoglobulin (Santa Cruz Biotechnology, Inc.).

Cell Proliferation and Cell Survival. Proliferation of U266 cells was determined by [3H]thyminde incorporation into DNA as described previously (30). Cells (15–20 × 10^4 cells/ml) were plated in 96-well microtiter plates in a total volume of 200 μl of RPMI 1640 supplemented with l-glutamine and 15% fetal bovine serum and incubated for the appropriate time at 37°C in the absence or presence of IL-6 and/or drugs. During the last 4 h of the incubation period, cells were labeled with [3H]thyminde (1.0 μCi/well) and harvested onto glass fiber filters. Incorporated radioactivity was determined by liquid scintillation counting. For recovery experiments, cells (20 ml) were incubated in T-75 flasks for 2 days with the appropriate agents, and 200-μl aliquots were removed for determining [3H]thyminde incorporation and cell viability by the trypan blue exclusion procedure (31). Another aliquot of 4 ml was removed for DNA distribution analyses as described below. The remaining cell suspension (14 ml) was centrifuged, supernatant was then removed, and the cell pellet was resuspended in medium alone. For control and IL-6-treated cells, the volume of medium used for resuspending the cell pellet was twice the original volume, whereas for cells treated with Taxol or vinblastine in the absence or presence of IL-6, a volume equivalent to the original amount was used for resuspending the cell pellet. Cells were then incubated at 37°C in T-75 flasks, and aliquots were removed on days 4 and 7 and analyzed for cell count, [3H]thyminde incorporation, and DNA distribution analyses. For calculating the number of viable cells and [3H]thyminde incorporation during the recovery period, values were multiplied by the appropriate dilution factor.

DNA Distribution Analysis. After treatment with appropriate agents, U266 cells were centrifuged, washed with normal saline, and fixed in 70% ethanol at 4°C. The fixed cells were stained with propidium iodide and RNase (32) and analyzed for DNA content on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, San Jose, CA). The percentage of cells containing <2N DNA relative to cells in the rest of the cell cycle was used to determine the sub-G1 peak (33). Cell-FITC cell cycle analysis software was used for determining the percentage of cells in the G1, S, and G2-M phases of the cell cycle, and the values were adjusted so that the percentage of cells in the sub-G1 fraction and in the G1, S, and G2-M phases totaled 100%.

Immunofluorescence Microscopy. Cytospin preparations of cells treated with drug and/or IL-6 for 2 days were fixed and stained with mouse antihuman α-tubulin (40 μg/ml in 0.2% BSA) or with a combination of mouse antihuman α-tubulin and rabbit antihuman erk-2 (2 μg/ml in 0.2% BSA). For staining with α-tubulin antibodies, cytospin slides were fixed for 30 min at room temperature in 3.7% formaldehyde in a buffer contain-
ing 0.1 mM 1,4-piperazine diethanesulfonic acid (pH 6.9), 1 mM magnesium sulfate, 2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and 2 mM glycerol; washed with PBS; and permeabilized with a mixture of ethanol and glacial acetic acid (2:1). For double immunostaining with antibodies to α-tubulin and erk-2, slides were fixed for 30 min at room temperature in 3.7% formaldehyde in PBS and 0.2% Triton X-100. The slides were then incubated with the primary antibodies (anti-α-tubulin alone, 37°C for 30 min; anti-α-tubulin and anti-erk-2, room temperature for 60 min), washed twice with PBS, and then incubated at room temperature for 1 h with the appropriate secondary antibodies [rhodamine-conjugated goat antimouse IgG F(ab')2 and FITC-conjugated antirabbit immunoglobulin]. After two washes in PBS, slides were mounted in 1 mg/ml p-phenylenediamine in 70% glycerol and PBS. Immunofluorescence was examined with a Nikon Eclipse microscope equipped with an epifluorescent attachment using the G-1A filter.

**RESULTS**

**Taxol and Vinblastine Inhibit Proliferation of U266 Cells.** Treatment of U266 human myeloma cells with Taxol or vinblastine for 2 days led to a dose-dependent inhibition (IC50 = 4.5 and 1.38 nM, respectively) of [3H]thymidine incorporation (Fig. 1). Because IL-6 serves as a major growth factor for myeloma cells in vivo and in vitro, we determined the effect of this cytokine on the inhibition of U266 cells by Taxol or vinblastine. Treatment of U266 cells with IL-6 (10 ng/ml) alone for 2 days led to stimulation of proliferation of these cells (53 ± 17.5%), as judged by [3H]thymidine incorporation (Table 1). IL-6 also increased (18 ± 7%) the number of cells in the S phase of the cell cycle (Table 1). However, IL-6 did not augment inhibition of [3H]thymidine incorporation by Taxol or vinblastine at the end of the 2-day treatment (Table 1).

**IL-6 Alters the DNA Distribution Pattern Induced by Taxol or Vinblastine.** Both Taxol and vinblastine altered the DNA distribution pattern of U266 cells. Taxol at the IC50 concentration did not lead to an arrest of cells in the G2-M phase of the cell cycle but led to an increase (3.6-fold) in the number of cells in the sub-G1 fraction (Tables 1 and 2). At higher concentrations (7.5 nM) of Taxol, which resulted in >70% inhibition of cell viability, an arrest of cells in the G2-M phase of the cell cycle was observed (data not shown). However, vinblastine led to a dose-dependent increase in the sub-G1 and sub-G1-M phase cell populations (Tables 1 and 2). The increase (2-fold) induced by vinblastine in the number of cells in the sub-G1 fraction was less than that induced by Taxol (3.6-fold).

Although IL-6 increased the proportion of cells in the sub-G1-M phase of the cell cycle for both Taxol and vinblastine treatment (Table 1), an increase (2.9-fold) induced by vinblastine in the number of cells in the G2-M phase was observed only for Taxol, but not vinblastine treatment (Tables 1 and 2). Maximum differences (65%) between Taxol- and IL-6 + Taxol-treated cells in the sub-G1 population were observed on day 7 of treatment (2 days of treatment + 5 days of recovery in medium alone).

**IL-6 Preferentially Enhances the Cytotoxicity of Taxol as Compared to Vinblastine.** To determine whether IL-6-induced changes in the DNA distribution pattern of cells treated with Taxol and vinblastine led to increased cytotoxicity, U266 cells were treated with drugs or IL-6 + drugs for 2 days and allowed to recover for an additional 5 days in medium alone. At the end of the recovery period, day 7 (2 days of treatment + 5 days of recovery), the survival fraction and [3H]thymidine incorporation of cells treated with drug + IL-6 was lower than that observed after treatment with drug alone (Fig. 2). However,
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IL-6-induced potentiation of the cytotoxicity of Taxol was significantly greater (P < 0.001 using the Bonferroni multiple comparison procedure) than that of vinblastine (Figs. 2 and 3). IL-6 decreased survival and [3H]thymidine incorporation of cells treated with Taxol by 52 ± 8% as compared to a 20 ± 8% decrease in cell survival and [3H]thymidine incorporation for cells treated with vinblastine. Furthermore, the kinetics of the IL-6-induced decrease in cell survival and [3H]thymidine incorporation for Taxol and vinblastine treatment differed (Fig. 3). Whereas the maximum differences between Taxol- and IL-6 + Taxol-treated cells were observed on day 7 of treatment, IL-6 maximally altered the cytotoxicity of vinblastine within 2–4 days.

IL-6 Enhances Taxol-induced Formation of Microtubule Asters and their Association with MAPK. Because IL-6 differentially altered the cytotoxicity of Taxol and vinblastine, we examined the effect of IL-6 on Taxol- and vinblastine-induced formation of microtubule asters. In addition, we evaluated the role of MAPK, a key enzyme involved in IL-6 signaling and in microtubule dynamics, in IL-6-mediated enhancement of the cytotoxic effects of Taxol and vinblastine. Immunostaining of U266 cells with mouse antihuman α-tubulin revealed the presence of microtubule asters in cells treated with Taxol (Fig. 4) or vinblastine (data not shown) in the absence or presence of IL-6. In the presence of IL-6, the number of cells containing microtubule asters increased by 2-fold for Taxol treatment (Table 3). In contrast, IL-6 did not alter the number of cells containing microtubule asters for vinblastine treatment (Table 3). Double immunostaining with antibodies to α-tubulin and erk-2 revealed identical staining patterns with both antibodies, indicating colocalization of the two proteins within the microtubule asters (Fig. 4).

DISCUSSION

Several therapeutic strategies for the treatment of multiple myeloma have focused on antagonizing the growth-promoting properties of IL-6, which has been implicated in the pathogenesis of this disease. Antibodies to IL-6 have been used in vitro and in vivo (9) to neutralize IL-6 activity, and, more recently, IL-6 superantagonists that block the IL-6 receptor in an inactive form have been designed (34). Other agents, such as dexamethasone, all-trans-retinoic acid, and IFN-α and -γ, have been shown to inhibit IL-6 production and/or down-regulate the IL-6 receptor (10, 13 15–18). However, the elevated in vivo levels of IL-6 frequently associated with multiple myeloma often limit the therapeutic efficacy of these agents. In this study, we evaluated the effect of two microtubule poisons, Taxol and vinblastine, in inhibiting the growth of U266 human multiple myeloma cells and the ability of IL-6 to alter this effect. Our results demonstrate that both drugs inhibit the growth of U266 cells, and that this effect is potentiated by IL-6, albeit to different extents. The combination of IL-6 and Taxol is significantly (P < 0.001) more potent than the combination of IL-6 and vinblas-
Fig. 4 Colocalization of MAPK with α-tubulin. Cytospin preparations of cells treated for 2 days in the absence (control; A) or presence of IL-6 (10 ng/ml; B), Taxol (5 nM; C and E), or IL-6 (10 ng/ml) + Taxol (5 nM; D and F) were fixed and stained with mouse anti-α-tubulin and anti-erk-2 antibodies. After two washes in PBS, slides were stained with rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated anti-rabbit IgG as described in "Materials and Methods." The stained cells were visualized with a fluorescent microscope. A–D, MAPK; E and F, α-tubulin.

tine, suggesting that the mechanisms involved in IL-6-mediated potentiation of the cytotoxicity of Taxol and vinblastine differ.

Whereas a simple mechanism involving recruitment of cells in the S phase of the cell cycle by IL-6 can account for potentiation of the cytotoxicity of vinblastine by IL-6, augmentation of the cytotoxicity of Taxol by IL-6 cannot be fully explained by this mechanism. This conclusion is based on the observation that the magnitude of increase in the cytotoxicity (determined by a decrease in [3H]thymidine incorporation and in the number of viable cells) of Taxol by IL-6 was greater (52 ± 8%) than the increase in the S phase of the cell cycle by IL-6 (18 ± 7%). In contrast, the increase (20 ± 8%) in the cytotoxicity of vinblastine by IL-6 was comparable to the IL-6-induced increase in the number of cycling cells. Furthermore, the time required to achieve maximal augmentation of the cytotoxicity of Taxol and vinblastine by IL-6 differed (day 7 versus day 4 after treatment, respectively). IL-6 also differentially modulated cell cycle phase changes induced by Taxol or vinblastine. Whereas IL-6 enhanced the number of cells in the S phase and G2-M phase of the cell cycle for both Taxol- and vinblastine-treated cells, an increase in the sub-G1 population was observed only for Taxol-treated cells. Thus, these findings indicate that regulation of the cytotoxicity of Taxol by IL-6 involves mechanisms in addition to augmentation of the S phase and G2-M phase of the cell cycle.

In U266 cells, Taxol increased the population of cells in the
sub-G₁ fraction (<2N DNA), which was further enhanced (65%) by IL-6. In contrast, the increase in the sub-G₁ fraction induced by vinblastine was not augmented by IL-6. Furthermore, maximum differences in the sub-G₁ cell population between Taxol- and IL-6 + Taxol-treated cells were not observed until day 7 (2 days of treatment, followed by 5 days of recovery in medium alone) of treatment. The similar kinetics of potentiation by IL-6 of Taxol-induced cytotoxicity and sub-G₁ cell population (representing cells that are damaged, including those damaged by apoptosis) suggests that a possible mechanism by which IL-6 enhances cell death by Taxol involves the up-regulation of apoptotic signaling pathway(s). However, Lumin and Sachs (35) reported that IL-6 and other growth factors, such as granulocyte macrophage colony-stimulating factor, inhibit apoptosis induced by several drugs, including the cell cycle-specific Vinca alkaloid vincristine in M1 cells. The effect of IL-6 on Taxol-induced apoptosis was not examined. Because apoptosis is activated via several mechanisms and at different phases of the cell cycle, it is possible that Taxol and vinblastine activate different apoptotic pathways. The relatively modest effect of Taxol on G₂-M phase arrest (Table 1), especially at lower concentrations, suggests that Taxol-treated cells probably exit mitosis after a brief delay in the S phase and G₂-M phase of the cell cycle. However, cells progressing through the cell cycle are likely abnormal and undergo cell death via apoptosis or some other mechanism. This event may be up-regulated by IL-6. In contrast, cells treated with vinblastine remain arrested in the G₂-M phase of the cell cycle. Because the effects of vinblastine are reversible, cells would be expected to recover after removal of the drug and progress normally through the cell cycle. It is possible that some cells arrested in G₂-M phase undergo apoptosis.

Both Taxol and vinblastine bind to tubulin; however, Taxol leads to irreversible polymerization of tubulin and stabilization of microtubules (22, 23), whereas vinblastine disrupts microtubule assembly (24). Thus, differences in the mechanisms by which these two drugs affect microtubule dynamics could, in part, explain the differential modulation of the cytotoxicity of these drugs by IL-6. An important intermediate involved in the regulation of microtubule dynamics and in the IL-6 signal transduction cascade is MAPK (36–40). MAPK, which exists as a cytosolic and microtubule-associated form, is activated following mitogenic stimuli as well as during differentiation. IL-6, a major proliferative signal in multiple myeloma, leads to activation of MAPK, whereas the effect of Taxol on MAPK activity is not clear. It has been demonstrated that the cytotoxic effects of Taxol are due to activation of MAPK, which in turn phosphorylates bcl-2 and inhibits its antiapoptotic activity (28, 41). In contrast, the report by Nishio et al. (42) showed that Taxol indirectly inactivates MAPK, which prevents phosphorylation of microtubule-associated proteins and their dissociation from tubulin, thereby leading to stabilization of tubulin polymerization. In our study evaluating the role of this enzyme in the cytotoxic effects of Taxol and vinblastine in combination with IL-6, we found that MAPK colocalized with α-tubulin to microtubule asters. The number of cells containing asters stained with antibodies to α-tubulin and erk-2 was greater in cells treated with IL-6 + Taxol than in cells treated with Taxol alone. However, differences between vinblastine- and IL-6 + vinblastine-treated cells were not observed. These results suggest that the cytoskeletal form of MAPK may play a role in the enhanced cytotoxic response of U266 cells observed for the combination of Taxol and IL-6. The mechanism by which this could occur is unknown at the present time, because the functional role of this form of the enzyme is poorly understood.

The differential regulation of the cytotoxicity of Taxol and vinblastine by IL-6 observed in U266 myeloma cells may represent a clinically relevant finding, thereby warranting evaluation of Taxol as a potential chemotherapeutic agent for the treatment of multiple myeloma. Because myeloma cells are often slow growing, and growth factors frequently antagonize drug-induced apoptosis (35), combination treatment with growth factors and several different cell cycle-dependent drugs has not proved clinically advantageous. However, the ability of IL-6 to potentiate Taxol-induced sub-G₁ fraction (apoptosis), in addition to enhancement of the S phase and G₂-M phase of the cell cycle, suggests that the combination of these two agents would be therapeutically more effective than the combination of nonspecific growth factors with other commonly used drugs. Furthermore, the frequent presence of IL-6 in the tumor microenvironment of multiple myeloma would be expected to enhance the cytotoxicity of Taxol, if it was used as a single agent.

REFERENCES


Interleukin 6 differentially potentiates the antitumor effects of taxol and vinblastine in U266 human myeloma cells.

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