A Potential Synergistic Anticancer Effect of Paclitaxel and Amifostine on Endometrial Cancer

Donghai Dai, Anna M. Holmes, Tan Nguyen, Suzy Davies, Daniel P. Theele, Claire Verschraegen, and Kimberly K. Leslie

Abstract

Although paclitaxel is one of the most effective chemotherapeutic agents, its usefulness is still limited in advanced and recurrent endometrial cancer. Amifostine protection of normal tissues against the side effects of chemotherapeutic agents has been clinically proven in cancer patients; however, its application in endometrial cancer has not been fully evaluated. We have investigated the use of paclitaxel and amifostine in controlling the growth of poorly differentiated endometrial cancer cells, HeC50co, in vitro and in vivo. Our studies show that amifostine had direct anticancer effects on endometrial cancer cells in vitro by arresting the cell cycle at the G1 phase and inducing apoptosis. Amifostine also inhibited s.c. tumor growth in athymic mice. Paclitaxel IC50 value was reduced from 14 to 2 nmol/L with pretreatment of a single dose of 178 µmol/L of amifostine for 72 hours. Amifostine also synergized with paclitaxel in the arrest of the cell cycle at the G2-M phase and in the induction of apoptosis. This two-drug regimen inhibited s.c. tumor growth as well as improved mouse survival significantly more than paclitaxel alone. Amifostine also significantly improved paclitaxel-induced cytotoxic effects on peripheral blood profiles. Our studies show that amifostine has direct anticancer effects on endometrial cancer. Our data have also shown a potential anticancer synergy between amifostine and paclitaxel in vitro and in vivo, whereas amifostine maintained a protective role in peripheral blood profiles. The dual specificity of amifostine action should be further investigated. (Cancer Res 2005; 65(20); 9517-24)

Introduction

Endometrial cancer is the fourth most common malignancy in women (1). The American Cancer Society estimated that there were 40,320 new cases and 7,090 deaths from endometrial cancer in 2004 (1). Currently, there is no effective therapy for advanced or recurrent endometrial cancer.

Paclitaxel is an antimitotube agent which is among the most potent single chemotherapeutic agents. Paclitaxel binds to the β-tubulin subunit and stabilizes the microtubules, resulting in disruption of normal microtubule dynamics during cell division (2). Failure of microtubule separation during the G2-M phase blocks cell mitosis and results in apoptosis. Application of this agent in endometrial cancer treatment is under investigation. A Gynecologic Oncology Group trial reports an overall response rate of 35.7% in advanced or recurrent endometrial cancer (3). The response rate is about 27.3% when paclitaxel is used in patients who have failed prior chemotherapy (4). The overall survival, objective response, and progression-free survival were improved by adding paclitaxel to the doxorubicin and cisplatin regimen (5). However, the median survival of patients treated with paclitaxel-based regimens is around 12 months. Unfortunately, the benefits of paclitaxel are also associated with serious adverse effects. These commonly include myelosuppression, gastrointestinal toxicity, and peripheral neuropathy, among others (5). It was reported that 62% of patients treated with paclitaxel had life-threatening leucopenia (3). In another study, where paclitaxel was used as a second-line chemotherapy (4), serious neutropenia occurred in more than 58% of patients with one treatment-related death. Improvement of outcome with the addition of paclitaxel to the doxorubicin and cisplatin regimen was accompanied by a much higher treatment-related death rate (5). Thus, it is imperative to search for more effective and safer chemotherapeutic regimens.

Amifostine (WR2721) was developed in 1979 as an antiradiation agent through the screening of 4400 chemicals by the Walter Reed Army Institute of Research (6). Since then, its protective effects against chemotherapy and radiotherapy in cancer patients have led to amifostine being FDA-approved as a cytoprotective agent against the adverse effects of chemotherapy and radiotherapy in normal tissues. Amifostine is a prodrug, which must first be converted to its active form, WR1065, by the enzyme alkaline phosphatase, which is located on the cell membrane. WR1065 is further metabolized through intracellular oxidation to the symmetrical disulfide WR33278, cysteamine, and mixed disulfides (7). The underlying mechanisms of amifostine protective effects are still not fully understood, but they are attributed to its ability to scavenge free radicals (8) and to its antimutagenic effects (9). This protection is based on the conversion of amifostine to the active form, WR1065, which is rapidly taken up into normal tissues (10). Amifostine dephosphorylation by membrane alkaline phosphatase occurs effectively in normal tissue, which has a suitable pH of 6.6 to 8.2 for alkaline phosphatase to work. The uptake of WR1065 is minimal in cancer cells because tumor tissue usually contains less membrane-bound alkaline phosphatase (11–14) and the environment of the tumors is more acidic, making the enzymatic activity less than optimal (15). Additionally, normal tissues contain the WR1065 transporter that allows active absorption against a concentration gradient (16). Taken together, these differences lead to a concentration of WR1065 that is about 50 to 100 times higher in normal tissue than in malignant tissue (16), thereby providing normal tissues with a remarkable protection against cytotoxic therapies.
There are reports that amifostine protects normal tissues without interfering with the chemotherapeutic agents used in cancer treatment (7). Concerns are still being raised, however, about amifostine protection in cancers (17) as well as any possible long-term adverse effects (18). We conducted the present studies to evaluate amifostine effect on the inhibition of endometrial cancer growth and its interaction with paclitaxel.

Materials and Methods

Cells and reagents. The poorly differentiated Hec50co endometrial cancer cell line was originally established from the ascites of a patient with poorly differentiated endometrial cancer (19) and has been reported in our previous publications (20–22). They are maintained in DMEM with 10% FBS and 95% O2 and 5% CO2 at 37°C. Amifostine (Ethylol, MedImmune Oncology, Inc., Gaithersburg, MD) was purchased from the University of New Mexico Hospital pharmacy and WR1065 was acquired from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Paclitaxel for the in vitro studies was purchased from Sigma (St. Louis, MO) and reconstituted in DMSO. Paclitaxel (Taxol, Mead Johnson, Princeton, NJ) for the in vivo study was purchased from the University of New Mexico Hospital pharmacy and further diluted in saline before i.v. administration.

Calculation of IC50. Hec50co cells were split into 24-well plates with equal cell numbers in each well and maintained in DMEM with 10% FBS. Cells were exposed to varying concentrations of paclitaxel (2–1,000 nmol/L), amifostine (28 μmol/L–5 μmol/L) or WR1065 (7 μmol/L–1 mmol/L) for different time periods as detailed in Results. Cells were harvested at the end of each study and counted under the microscope using a hemocytometer. The number of surviving cells was expressed as a percentage of the control cells. The IC50 values were calculated using SigmaPlot (Systat Software, Point Richmond, CA) sigmoidal dose response equation with variable slopes. Means and SEs were calculated from at least three experiments. The combination index was calculated using the following equation: combination index = (Am)50 / (Am)50 + (Bm)50 / (Bm)50 where (Am)50 is the concentration of drug A necessary to achieve a 50% inhibitory effect in the combination; (Am)50 is the concentration of the same drug that will produce the identical level of effect by itself; (Bm)50 is the concentration of drug B that will produce a 50% inhibitory effect in the combination; and (Bm)50 is the concentration of drug B that will produce the same level of effect by itself. Combination index > 1 indicates antagonism; combination index < 1 indicates synergy; and combination index = 1 indicates an additive effect (23).

Flow cytometry. Cells were harvested after treatment with different regimens and pelleted by centrifugation for 5 minutes at 500 × g. Cells were resuspended in 1 mL of Krishan’s solution containing propidium iodide, NP40, and RNase. Cells were analyzed by a FacScan machine (Becton Dickinson, Franklin Lakes, NJ) at the University of New Mexico Flow Cytometry Core Facility and DNA histograms were prepared and analyzed by the CellQuest Program version 3.3 (Becton Dickinson) to provide fits for the G0-G1, S, and G2-M fractions of the population. Means and SEs were calculated from at least three experiments.

Apoptosis assay. Hec50co cells were grown in six-well plates and incubated with various drugs at different time points as indicated in Results. They were washed with PBS and incubated for 5 minutes with staining solution containing 3.7% formaldehyde, 0.6% NP40, and 11 μg/mL Hoechst 33342. Apoptotic cells were confirmed by their fragmented nuclear morphology and counted under the microscope with a 4′,6-diamidino-2-phenylindole (DAPI) filter. The ratio was obtained as a percentage of apoptotic cells over total cell number in one high magnification field. Means and SEs were calculated from at least three experiments.

Creation of xenograft endometrial tumors and drug treatment regimens in athymic mice. Sc. and ip. xenografts were created as reported previously (24). Briefly, Hec50co cells were grown in cell culture medium, DMEM with 10% FBS, until subconfluence and then harvested by trypsinization. Six- to eight-week-old athymic, CrlNU/NU-nuBR female mice were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocols were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. The s.c. xenografts were created through injection of 5 million Hec50co cells in 100 μL DMEM. The tumors were measured weekly and tumor cross-sectional areas (mm2) were calculated using the following formula: length (mm) × width (mm) × π / 4. The l.p. tumors were created through i.p. injection of 10 million Hec50co cells (24). For the investigation of amifostine effect on s.c. tumors, amifostine (60 mg/kg in saline) was given i.p. thrice a week for 3 weeks. The control group received only saline. To investigate the effects of paclitaxel and amifostine on s.c. tumor growth, amifostine (120 mg/kg) was given through i.p. injection every Monday, Tuesday, and Wednesday starting 2 weeks after s.c. cancer cell inoculation and continuing for 4 weeks. Paclitaxel (12 mg/kg) was given through tail-vein injection 30 minutes after amifostine every Wednesday for 4 weeks. Tumors were isolated 1 week after the last dose of paclitaxel and wet weights were analyzed by one-way ANOVA.

To study paclitaxel and amifostine effect on the survival of mice bearing l.p. tumors, amifostine (120 mg/kg) was given through s.c. injection every Monday, Tuesday, and Wednesday starting 2 weeks after cancer cell inoculation and continuing for 4 weeks. Paclitaxel (12 mg/kg) was given through tail-vein injection 30 minutes after amifostine every Wednesday. The control group received only vehicle. A daily score for each mouse was documented by an animal facility technician without knowledge of treatment regimens. The technician euthanized any animal when a moribund condition was observed which met the definition outlined in the Animal Health Monitoring Criteria in the animal use protocol. The time from cancer cell transplantation to euthanasia was documented. All deceased animals were surgically examined and occurrences of advanced i.p. tumors were confirmed. The data were analyzed by the Kaplan-Meier survival analysis with log-rank test. All pairwise comparisons were analyzed by the Holm-Sidak method.

Study of the peripheral blood profile. In conjunction with the survival study described above, peripheral blood profiles were investigated. Blood was collected from mouse saphenous veins 1, 2, 3, 5, 7, 9, and 12 days after the last paclitaxel injection. The RBC, after dilution in EDTA solution, were counted using a hemocytometer under the microscope. The total WBC component was similarly counted after incubation with a lytic solution containing 0.01% crystal violet and 3% acetic acid. Blood smears were made on a glass slide and stained using a Hema 3 staining kit (Fisher, Pittsburgh, PA). Absolute neutrophil counts were obtained using the following formulaic percentage of neutrophils over total WBC × 100.

The platelet count was derived from the ratio of platelets over RBC × the RBC count. All data were expressed as their relative value to the control. Student’s t test was used to compare blood counts between the control and any treatment group at a specific time point, as well as between animals treated with paclitaxel alone and those treated with both drugs.

Western blotting. Primary antibodies for c-Jun, JunB, JunD, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–poly(ADP-ribose) polymerase antibody was purchased from EMD Biosciences (San Diego, CA). Extracts containing 100 μg of protein per lane were resolved on 12.5% SDS-PAGE gels as previously described (22). Proteins were transferred to nitrocellulose membrane, probed with primary

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<th>Table 1. The IC50 values of paclitaxel and amifostine</th>
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<td>Paclitaxel (pretreated with 178 μmol/L amifostine)</td>
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antibodies, and visualized using enhanced chemiluminescence. β-Actin was used as a loading control.

Methods of statistical analysis were detailed in Results and figure legends.

Results

Direct anticancer effect of amifostine on endometrial cancer cells in vitro. Amifostine inhibits endometrial cancer cell growth in vitro. For Hec50co cells, the IC_{50} for amifostine was 356 ± 63 μmol/L when cells were exposed to a single dose for 72 hours (Table 1). Inhibition of cell growth was 4%, 24%, and 50% when cancer cells were exposed to the IC_{50} dose of amifostine for 24, 48, and 72 hours, respectively (Fig. 1A). The difference in cell growth between control and 24-hour treatment or between 24- and 48-hour treatment was not statistically significant, suggesting that 48-hour incubation with amifostine is necessary to achieve a direct anticancer effect. When WR1065, the active metabolite of amifostine, was used, the IC_{50} was 78 ± 4 μmol/L after a 72-hour treatment (Table 1). As with amifostine, WR1065 also requires 48 hours to show significant inhibition of endometrial cancer cell growth (Fig. 1A). The IC_{50} of WR1065 is 1/5 of amifostine, consistent with the reported ratio of plasma concentration of WR1056 and amifostine in patients after amifostine infusion (25), suggesting that conversion of amifostine to WR1065 and other metabolites has functional relevance.

Amifostine also inhibits cancer cell cycling (Fig. 1B). The percentage of cells in the G1 phase increases gradually from 52% in controls to 54.4%, 58.9%, and 61% in cells treated with a single dose of 356 μmol/L amifostine for 24, 48, and 72 hours (Fig. 1B). A one-way ANOVA followed by paired Tukey’s analysis showed that all pairwise comparisons were different except the 24-hour amifostine versus control. This indicates that amifostine antiproliferative action requires prolonged time to take effect. Amifostine has a slight effect on the G 2-M phases at 24 and 48 hours. However, this effect disappears by 72 hours. Thus, the major effect of amifostine on the cell cycle is to delay cancer cell transition from the G1 to S phase.

Amifostine induced apoptosis in Hec50co endometrial cancer cells. Hec50co cells showed a time-dependent induction of apoptosis after incubation with a single dose of 356 μmol/L amifostine (Fig. 1C). The percentage of apoptotic cells increased from 2% in controls to 50% after a 72-hour amifostine treatment. Again, the difference between controls and 24-hour amifostine, as well as between 24 and 48 hours, is not significant according to one-way ANOVA followed by Tukey’s analysis.

Figure 1. Time-dependent inhibition of Hec50co cell growth by amifostine and WR1065. A, time-dependent growth inhibition. Saline or 356 μmol/L amifostine or 77 μmol/L WR1065 was added for 24 (24H), 48 (48H), and 72 (72H) hours. Surviving cells were counted with a hemocytometer under a microscope. Cell numbers were expressed as a percentage of their controls. All comparisons were different, except control versus 24 hours, by one-way ANOVA followed by Tukey’s analysis (P < 0.05). B, time-dependent inhibition of the cell cycle. Cells grown in culture dishes were treated with saline or 356 μmol/L amifostine for 24, 48, and 72 hours. Changes in the percentage of cells at G1 or G2-M phases of the cell cycle were analyzed by one-way ANOVA followed by Tukey’s analysis for comparison. All comparisons of G1 cell phase were different except control versus 24 hours and 48 versus 72 hours (P < 0.05). C, time-dependent induction of apoptosis. Cells were treated with saline or 356 μmol/L amifostine for 24, 48, and 72 hours. Apoptotic cells were confirmed by their fragmented nuclear morphology and counted under the microscope with a DAPI filter. The ratio was obtained as a percentage of apoptotic cells over total cell number in the same field. Changes in the percentage of apoptotic cells were analyzed by one-way ANOVA followed by Tukey’s analysis for comparison. All comparisons were different except control versus 24 hours, 24 versus 48 hours, and 48 versus 72 hours (P < 0.05).

Figure 2. Amifostine inhibition of s.c. tumor growth. Hec50co cells were transplanted s.c. into athymic mice. Amifostine was given at 60 mg/kg through i.p. injection, thrice a week for 3 weeks. Tumor cross-sectional areas (mm²) were measured weekly. Points, mean (n = 12 each); bars, SE. X axis, time after cancer cell inoculation.
Amifostine inhibition of xenografted endometrial cancer growth in athymic mice. Amifostine effect on in vivo tumor growth was tested in athymic mice. Poorly differentiated Hec50co endometrial cancer cells were injected s.c. into athymic mice and the cross-sectional area of s.c. tumors was measured with a caliper once a week. Amifostine was given at a dose of 60 mg/kg through i.p. injection thrice a week for 3 weeks starting on the day of cancer cell inoculation. The control group received only vehicle. The average tumor size in amifostine-treated animals (61 mm²) was found to be significantly smaller than the control (118 mm²) at the end of the study (Fig. 2, n = 12 each, P < 0.05, t test). Therefore, our data support a direct inhibitory effect of amifostine on endometrial tumor growth in vivo.

Amifostine enhances paclitaxel anticancer effect in vitro. The IC₅₀ for paclitaxel with 24-hour incubation was 14 ± 1.7 nmol/L (Table 1). The IC₅₀ of paclitaxel was reduced to 2 nmol/L when Hec50co cells were pretreated with a single dose of 178 μmol/L amifostine for 48 hours before incubation with paclitaxel for 24 hours. The combination index was 0.62 (Table 1), suggesting a synergistic effect between these two drugs.

As expected, paclitaxel blocks the endometrial cancer cell cycle at the G2-M phase. Paclitaxel treatment increases the percentage of cells at G2-M phase from 18.3% in controls to 47.9% (Fig. 3A). Addition of amifostine to paclitaxel treatment significantly increased the percentage of cells in G2-M phase to 59% although amifostine alone did not have an effect on the G2-M phase at 72 hours (Figs. 1B and 3A).

Amifostine also enhanced paclitaxel effect on the induction of apoptosis (Fig. 3B). The percentage of apoptotic cells was 22.7% with paclitaxel and amifostine. This is higher than the sum of paclitaxel alone (7.3%) and amifostine alone (12.3%) even when a reduced dose (1/7 of paclitaxel and 1/2 of amifostine) was used. Apoptosis involves activation of caspases to execute the death program. Among the targets is poly(ADP-ribose) polymerase, which is cleaved to generate a signature 85 kDa apoptotic fragment. Our immunoblot study showed that the level of 85 kDa fragment was remarkably increased in cell extracts treated with paclitaxel, amifostine, or both (1/7 paclitaxel and 1/2 amifostine) as compared with the control (Fig. 3C), suggesting that combination treatment can effectively induce apoptosis with reduced doses.

Amifostine synergistic effect with paclitaxel on cell growth is time dependent (Fig. 3D). When cells were incubated simultaneously with paclitaxel and amifostine for 24 hours, cell growth was not significantly different than treatments with paclitaxel alone. However, pretreatment with amifostine for 24 hours resulted in significantly more growth inhibition than either paclitaxel alone or simultaneous treatment. More remarkable inhibition was observed in cells pretreated with amifostine for 48 hours before paclitaxel (Fig. 3D).

Amifostine enhances paclitaxel inhibition of subcutaneous tumor growth in athymic mice. Paclitaxel has been proven to be an effective therapeutic agent against human endometrial cancer. We tested the effect of paclitaxel on the control of xenografted endometrial tumor growth. We also investigated the effect of
Amifostine significantly enhances the effect of paclitaxel on the survival of animals with advanced endometrial cancer. We conducted a survival study of paclitaxel and amifostine in an i.p. xenograft model (24), which should better simulate advanced or recurrent endometrial cancer in patients. Paclitaxel was given at a dose of 12 mg/kg every Wednesday and amifostine was administered at 120 mg/kg every Monday, Tuesday, and Wednesday. Treatment started 2 weeks after i.p. inoculation of Hec50co cancer cells and continued for 4 weeks. The paclitaxel group had a median survival of 7.9 weeks [95% confidence interval (95% CI), 6.9-11.8], significantly longer than the control group (median, 5.4 weeks; 95% CI, 5.1-7.1). Additionally, the animals receiving both paclitaxel and amifostine had a median survival of 13.9 weeks (95% CI, 11.4-16.5) and lived significantly longer than animals in the control group or those treated with paclitaxel alone (Fig. 5). Thus, adding amifostine to a paclitaxel regimen has significant benefit on the survival of mice with advanced tumors.

Amifostine improves peripheral blood profiles in animals treated with paclitaxel. To investigate amifostine protection against paclitaxel cytotoxic effect on blood profiles in animals, we collected peripheral blood 1, 2, 3, 5, 7, 9, and 12 days after the last dose of paclitaxel in conjunction with the survival study described above. Paclitaxel and amifostine had already been given to these mice for four cycles, and some mice showed advanced tumor progression. Paclitaxel had suppressive effects on peripheral blood profiles (Fig. 6). The RBC count declined on the 2nd day and reached their nadir on the 3rd day, and recovered on the 9th day after paclitaxel treatment. Addition of amifostine has a significant effect on the WBC count on the 3rd, 5th, and 7th day. Overall, the WBC reading in animals with the two-drug regimen was comparable to the controls.

Amifostine and Paclitaxel in Endometrial Cancer

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Amifostine on paclitaxel control of tumor growth (Fig. 4). Paclitaxel and amifostine treatment was begun about 2 weeks after s.c. cancer cell implantation. Amifostine was given at a dose of 120 mg/kg every Monday, Tuesday, and Wednesday through i.p. injection, and paclitaxel was given at a dose of 12 mg/kg every Wednesday through tail-vein injection. The control animals received only vehicle. The treatment was repeated every week for 4 weeks. The wet weights of isolated tumor (mg) were obtained. Differences in tumor weight at the end of the study were analyzed by one-way ANOVA followed by Tukey's analysis for comparison.

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Amifostine and Paclitaxel in Endometrial Cancer

Figure 4. Paclitaxel and amifostine effect on tumor weight (mg). Hec50co cells were transplanted s.c. into athymic mice. Treatment with vehicle, paclitaxel, and both paclitaxel and amifostine started 2 weeks after cancer cell transplantation and continued for 4 weeks. Paclitaxel at 12 mg/kg i.v. was given once a week. Amifostine at 120 mg/kg i.p. was given thrice a week. The wet weights of isolated tumor (mg) were obtained. Differences in tumor weight at the end of the study were analyzed by one-way ANOVA followed by Tukey's analysis for comparison.

Figure 5. Paclitaxel and amifostine effects on mouse survival. Athymic mice were transplanted i.p. with human endometrial cancer cells, Hec50co. Two weeks later, these animals were given vehicle or paclitaxel (12 mg/kg i.v., 3rd day every week) or both paclitaxel and amifostine (120 mg/kg s.c., first 3 days every week) for 4 weeks. A daily score for each mouse was documented by an animal facility technician without knowledge of treatment regimens. The technician euthanized any animal when a moribund condition was observed which met the definition outlined in the Animal Health Monitoring Criteria in the animal use protocol. The time from cancer cell transplantation to euthanasia or death was documented. The data were analyzed by Kaplan-Meier survival analysis with log-rank test. All pairwise comparisons were analyzed by the Holm-Sidak method.

Survival Analysis

Table 1. 

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<td>10</td>
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<tr>
<td>Paclitaxel</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Paclitaxel + Amifostine</td>
<td>4</td>
<td>12</td>
<td>16</td>
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The neutrophil counts were consistently low in animals treated with paclitaxel, probably because four cycles of paclitaxel treatment had a more severe adverse effect on neutrophils than the other cells. The neutrophil count was less than half of the control level on the 3rd day and did not recover by 12 days after paclitaxel treatment. These animals did not seem to suffer from infections. This is likely due to their being housed in a pathogen-free facility specifically designed for immunocompromised mice. However, an effect of neutropenia on mouse survival cannot be excluded. The two-drug regimen significantly improved neutrophil counts on the 3rd, 5th, and 7th day.

Platelet counts were less affected compared with the other blood cells. This component remained unchanged except on the 3rd and 5th day after paclitaxel treatment. Amifostine did not have any significant effect on platelet count.

Paclitaxel and amifostine regulation of c-Jun, JunB, and JunD proteins. Oncogene c-jun has been shown to be overexpressed in the human endometrial cancer cell line Hec50co (22). This oncogene plays a crucial role in the support of cancer cell growth in vitro and in vivo. Progestin inhibits endometrial cancer growth through regulation of AP-1 transcription factors (22). Hec50co cells were treated with vehicle, 356 μmol/L amifostine for 72 hours, 14 nmol/L paclitaxel, or both amifostine (178 μmol/L for 72 hours) and paclitaxel (2 nmol/L for 24 hours). When 100 μg of protein extracts from these treated cells were used for SDS-PAGE, our immunoblot results showed that the c-Jun protein had been down-regulated by paclitaxel alone, amifostine alone, or both paclitaxel and amifostine with equal potency (Fig. 7). Because paclitaxel and amifostine were used in highly reduced doses in the combination, this suggests that paclitaxel and amifostine work together more effectively in the down-regulation of c-Jun proteins.

Amifostine alone had no effect on the JunB protein level. Paclitaxel alone or with amifostine reduced JunB protein levels. Interestingly, JunD was up-regulated by combinational treatment, whereas neither paclitaxel nor amifostine alone had any effect. These results suggest that AP-1 factors could be potential targets for paclitaxel and amifostine anticancer effects.

Discussion

The use of amifostine to treat patients with endometrial cancer was recently reported (26). However, this report described a phase II trial to evaluate the response rate and survival in patients treated with paclitaxel, carboplatin, and amifostine. Although this regimen is well tolerated and showed significant activity in advanced, recurrent, and refractory endometrial cancer, the benefit of amifostine could not be clearly defined. There are numerous reports supporting the observation that amifostine protects normal tissues without compromising the antitumor effects of chemotherapeutic agents (7). However, amifostine has also been reported to potentially compromise anticancer therapies. Previous research has indicated that amifostine impaired p53-induced apoptosis in myeloid leukemia cells (17) and potentiated bleomycin-induced DNA damage in human lymphocytes (27). Pretreatment with...
amifostine was associated with increased doxorubicin toxicity and a higher mortality, which seems to be dose-dependent (18). Thus, it is critical to define the effect of amifostine on endometrial cancer. We have conducted a series of in vitro and in vivo studies to investigate the therapeutic effects of amifostine alone and in combination with paclitaxel on this disease. We have shown that amifostine does not protect tumors from paclitaxel effects; instead, it enhances the cytotoxic effect on cancer cell growth.

Our data support a direct inhibitory effect of amifostine on endometrial cancer (Table 1). We found that amifostine did not have a significant effect at 24 hours, and at least 48 hours are needed to achieve significant growth inhibition (Fig. 1). This time line seemed to be different from amifostine protective action in normal tissues as reported by others (7). It will be important to investigate whether different mechanisms are responsible for amifostine anticancer effects and its protection of normal tissues.

We have previously reported that the oncogene c-jun is necessary for the constitutive growth of endometrial cancer cells, and that progesterone inhibitory effects may be mediated through its regulation of c-Jun and other AP-1 transcription factors (22). Indeed, direct interactions between c-Jun and WR1065 have been reported (28). It is tempting to speculate that amifostine (or WR1065) may inhibit cancer cell growth through interference of c-Jun activity. Our studies did show the differential regulation of c-Jun, JunB, and JunD proteins by paclitaxel and amifostine (Fig. 7). Thus, mediation of amifostine anticancer effect by AP-1 factors could be a potential mechanism in our model and requires further study.

Amifostine direct anticancer effect was confirmed in vivo using a xenograft endometrial cancer model. Amifostine has been shown to significantly inhibit s.c. tumor growth in athymic mice (Fig. 2). Amifostine has also been shown to be synergistic with paclitaxel in the inhibition of cell growth (Table 1), in the arrest of the cell cycle at the G2-M phase (Fig. 3A), and in the induction of apoptosis (Fig. 3B). As a combinational treatment, dosing and scheduling are important issues. We have shown that a single dose of 1/2 of amifostine and 1/7 of paclitaxel IC50 values can achieve a 50% growth inhibition (Table 1). Thus, adding amifostine to a paclitaxel regimen will significantly reduce the required dose of paclitaxel. Amifostine is usually well tolerated and recent reports of s.c. administrations of amifostine may further reduce adverse effects (29, 30). In fact, s.c. administration of amifostine in our survival study was effective in the enhancement of paclitaxel anticancer effects and protection of peripheral blood cells (Figs. 5 and 6). As with amifostine alone, synergistic growth inhibition requires administration of amifostine at least 24 hours before paclitaxel to provide extra benefit (Fig. 3D). The in vivo studies (Figs. 5 and 6) also suggest a potential synergistic anticancer effect between paclitaxel and amifostine, although amifostine was not tested alone in the same experiment and statistical analysis for the synergistic effect was not possible. Most clinical studies reported use of amifostine 15 to 30 minutes before chemotherapy as necessary for amifostine cytoprotection (26, 29, 31). We could not determine whether a specific treatment schedule is necessary for the cytoprotective or anticancer effect of amifostine because we administered three doses of amifostine 48, 24, and 0.5 hours before paclitaxel, respectively (amifostine was given on Monday, Tuesday, and Wednesday whereas paclitaxel was given 30 minutes after amifostine on Wednesday).

The mechanism of amifostine enhancement of anticancer effects of chemotherapeutic agents is not well understood. Amifostine induced a G1 arrest and protected against paclitaxel toxicity in cells with functional p53 (32). In contrast, amifostine enhanced the cytotoxicity of paclitaxel in the absence of p53. We know that p53 is mutated and the protein is absent in Hec50co cells.4 Our speculation is that in normal tissues, such as bone marrow, amifostine inhibition of G2-S cell cycle transition with a functional p53 gene will be protective against paclitaxel cytotoxicity. Shen et al. (28) reported that WR1065 protection against paclitaxel in murine embryo fibroblasts is p53 dependent. Thus, different genetic backgrounds in malignant and normal tissues may provide a basis for the selective effects of amifostine in these tissues.

Our studies provide evidence supporting the testing of amifostine in paclitaxel-based regimens in human endometrial cancer patients. Based on our findings, the combination of paclitaxel and amifostine may offer benefits over paclitaxel alone in the control of cancer growth. Amifostine protection of the hematologic system can be further assessed. We realize that an IC50 value of 356 μmol/L of amifostine in vitro is not a small dose. However, an i.v. infusion of amifostine at a dose of 740 mg/m2 could produce a plasma Cmax of about 240 μmol/L (25), which is well over the 178 μmol/L that we tested for a synergistic effect with paclitaxel. Thus, an effective dose is achievable in humans.

A synergistic effect between amifostine and paclitaxel has not been reported in most clinical and preclinical studies involving these two drugs. This may be due to the uniqueness of endometrial cancer. The other possibility is that amifostine was not administered at least 24 hours before paclitaxel. The effectiveness of amifostine was often tested when it is administered no more than 30 minutes before chemotherapeutic agents in vitro and in vivo (26, 29, 31, 33). Although a comparison of the phase II trial involving paclitaxel, carboplatin, and amifostine (26) with other chemotherapies without amifostine should be made with caution.

4 C.A. Hu, et al., personal communication.
the regimen tested did show a response rate and survival rate very similar to a regimen with doxorubicin, cisplatin, and paclitaxel. The hematologic toxicity was well tolerated without growth factor (Filgrastim) support and no treatment-related death was reported. Additionally, the HeC50co cell line we used was derived from a poorly differentiated human endometrial cancer. It has been shown that HeC50co cells have lost p53 activity due to mutations and are negative for estrogen and progesterone receptors, and develop papillary serous morphology in vivo, suggesting that xenografts created from this cell line have features of type II human endometrial cancer. Thus, paclitaxel plus amifostine may have a particular application in this subpopulation of endometrial cancer. Our studies support that mechanisms of amifostine action should be investigated further to explore its dual specificity in malignant and normal tissues.

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References

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