Effects of sustained and intermittent paclitaxel therapy on tumor repopulation in ovarian cancer

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Abstract

Tumor repopulation between cycles of chemotherapy likely has a negative effect on clinical outcome in ovarian cancer patients. Thus, avoiding treatment-free periods when tumor cells proliferate by providing sustained chemotherapy regimens may improve clinical response. We investigated the effect of sustained versus intermittent paclitaxel administration on tumor repopulation in ovarian cancer. Growth, clonogenic survival, and apoptosis were followed in SKOV3 and A2780 cells after equivalent exposure to intermittent and sustained levels of paclitaxel. *In vivo* tumor repopulation in response to sustained and intermittent paclitaxel therapy was investigated in an i.p. xenograft model of human ovarian cancer. Tumor growth, proliferation, and apoptosis were evaluated at different intervals during and after the course of treatment using 5-bromo-2-deoxyuridine uptake, caspase-3, and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling immunoassays. Sustained treatment significantly reduced survival *in vitro* in both cell lines, whereas an increase in clonogenic survival was observed in the intermittent group with each treatment gap, indicating a gradual acceleration in repopulation rates. Similarly, *in vivo*, sustained therapy resulted in a significant reduction of tumor growth and proliferation. Intermittent therapy resulted in increased tumor proliferation and no efficacy. The percentage of apoptotic tumor cells significantly increased in the sustained group, whereas no significant changes were seen in the control and intermittent groups. Intermittent administration of paclitaxel significantly augmented both *in vitro* and *in vivo* tumor repopulation rates, whereas sustained delivery inhibited tumor growth and repopulation. Sustained administration of paclitaxel may increase chemoresponsiveness and clinical response in ovarian cancer by attenuating tumor repopulation. [Mol Cancer Ther 2008;7(3):630–7]

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and remains the fourth leading cause of cancer-related deaths among women (1, 2). The first-line treatment involves cytoreductive surgery followed by courses of i.v. combination taxane/platinum chemotherapy generally consisting of paclitaxel and carboplatin. Although the initial response rate is ~70% to 80%, the majority of patients relapse within relatively short periods (1, 3, 4). This may be attributed to high intrinsic and/or acquired tumor drug resistance in combination with repopulation of surviving tumor cells in between sequential treatment intervals (1, 5).

Because most chemotherapeutic agents target proliferating cells, tumor cells near blood vessels are most likely to be killed with each treatment because of their high proliferation rates and enhanced drug access. Following the death of these cells, nutrition of the more distant cells within the tumor mass improves and their cell death decreases; they reenter the cell cycle and repopulate the tumor (6). Indeed, an increased rate of tumor repopulation has been observed during courses of fractionated radiation therapy and the implementation of continuous low-dose radiation schedules has resulted in substantial improvements in local tumor control and patient survival (7). As intervals between cycles of chemotherapy are much longer than radiation regimens, it is very likely that tumor repopulation will have a major role on clinical outcome.

In this regard, strategies such as sustained and localized chemotherapy may have the potential to improve response to chemotherapy. Localized administration will provide high regional drug concentrations, whereas sustained chemotherapy will avoid treatment-free periods when tumor cells repopulate. As peripheral tumor cells are killed, local and sustained drug concentrations will penetrate the next tumor cell layer resulting in further cell death. Moreover, systemically available drug will also penetrate the tumor through the vasculature. This strategy may be the ideal scenario for the treatment of residual disease postoperatively and ultimately in the prevention of recurrence. Further investigation is required on repopulation rates following chemotherapy and in between cycles of chemotherapy and optimal dosing intervals. To date, there are virtually no studies that examine the effects of tumor repopulation during courses of chemotherapy in ovarian cancer. Therefore, we investigated the effects of sustained and localized paclitaxel chemotherapy on ovarian tumor repopulation and responsiveness.

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Materials and Methods

Cell Lines/Cell Culture

The SKOV3 human ovarian adenocarcinoma cell line was obtained from the American Type Culture Collection and the A2780 human ovarian carcinoma cell line was kindly provided by Dr. Michael Wiese (University of Bonn). Cells were grown in monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2.

Drug Preparation and Delivery

The commercially available formulation of paclitaxel, Taxol (6 mg/mL), was purchased from Bristol-Myers Squibb. For the *in vitro* studies, Taxol was dissolved in cell medium to achieve the desired drug concentrations. Cremophor EL and dehydrated ethanol (1:1, v/v) vehicle from Sigma was also used as control in the same fashion in addition to nontreated controls. For the *in vivo* xenograft studies, paclitaxel was administered i.p., either as bolus injections diluted in sterile 0.9% NaCl (200 μL total volume) or as sustained from surgically implanted Alzet osmotic pumps purchased from Durect. Alzet pumps were loaded with drug according to the manufacturer's instructions. Cremophor EL/ethanol vehicle groups were not included as controls due to high toxicity and mortality rates (8).

Effects of Sustained and Intermittent Chemotherapy on Ovarian Cancer Cells

Exponentially growing SKOV3 and A2780 cells (1×10^{5}) were exposed to different paclitaxel concentrations ranging from 0 to 20,000 nmol/L for 24 h. Cell viability was determined by the clonogenic assay. Briefly, following treatment, cells were washed three times with PBS, trypsinized, counted and then seeded in serial dilutions, and incubated for 7 days; subsequently, resulting colonies were stained with 1% crystal violet. Colonies containing >50 cells were considered as viable. Plating efficiencies (PE) were calculated as [(number viable colonies) ÷ (number cells plated)] and the number of clonogenic cells was calculated based on the PE [(number cells plated$_{\text{at each time point}}$) × PE]. The fraction of surviving cells was determined by the ratio of the $PE_{treated}$ and $PE_{control}$ cells. The IC_{80-90} value was selected for the intermittent treatment concentration as typical clinical cycles of chemotherapy result in ~80% to 90% tumor cell kill. The sustained treatment concentration was calculated based on the AUC for the intermittent exposure. Briefly, 10^6 cells were plated in 50 mL flasks (n = 3) and exposed to the chosen concentrations with appropriate controls. Cells were equilibrated with CO_2 and incubated at 37°C. Intermittent treatments consisted of a 24-h paclitaxel exposure once a week for a total of three cycles, whereas cells receiving the sustained treatment had their medium replaced with fresh drug-containing medium once a week. The number of cells in each group was determined before and after each treatment cycle (same time point chosen for control, intermittent, and sustained) and clonogenic assays were done at these respective time points to determine the extent of cell survival. Experiments were done in triplicate.

Effects of Paclitaxel Treatment Duration on *In vitro* Caspase Activation and Cell Survival

Because apoptosis is the major form of cell death in response to paclitaxel and an indicator of treatment cytotoxicity, we investigated the degree of caspase activation and cell survival at various paclitaxel concentrations and exposure times in the SKOV3 and A2780 ovarian carcinoma cell lines. Briefly, 4×10^4 cells per well were seeded in 96-well microplates and incubated with 0 to 20,000 nmol/L paclitaxel-containing medium for 24, 48, and 72 h at 37°C and 5% CO_2. Following each treatment point, cells were incubated with cell lysis buffer and DEVD-rhodamine-110, a specific substrate for caspase-2, caspase-3, and caspase-7 (caspase-6, caspase-8, caspase-9, and caspase-10 detected to a lesser extent), for 2 h (Homogeneous Caspase Assay; Roche). On cleavage of the substrate by activated caspases, fluorescence of released rhodamine-110 was measured. Relative fluorescence units (RFU) were converted to free rhodamine-110 (9) via the standard curve and the induction factor of caspase activity was calculated as a ratio of $RFU_{\text{treated cells}} \div RFU_{\text{untreated cells}}$. The increase in caspase activity was defined as the $RFU_{\text{treated cells}}$ - $RFU_{\text{untreated cells}}$. Cell viability was determined by clonogenic assays.

Generation of Xenografts

Female CD-1 immunocompromised mice (4-6 weeks old, 18-20 g), purchased from Charles River, were injected i.p. with 1×10^7 SKOV3 cells and suspended in 200 μL RPMI 1640 (Invitrogen). This tumor model closely mimics the clinical presentation of ovarian cancer as described previously (8, 10). Animals were housed under sterile conditions in microisolator cages, fed standard chow diet with water *ad libitum*, and maintained on an automatic 12-h light cycle at 22°C to 24°C. All studies were conducted in accordance with the guidelines of the Canadian Animal Care Council.

Treatments

Tumor-bearing mice received paclitaxel (Taxol; 60 mg/kg total dose over 3 weeks) either as intermittent therapy (i.p. bolus doses of 20 mg/kg on a q7d × 3 schedule) or as sustained therapy (Alzet pumps surgically implanted i.p. providing sustained delivery of 20 mg/kg/wk) and were compared with nontreated controls (n = 20 per group). Treatment was initiated 14 days after SKOV3 inoculation, referred to as day 0. Body weight was monitored weekly to assess treatment related toxicities and disease progression. Animals were followed for a total of 6 weeks (due to severe disease progression) and this time point was set as the survival time endpoint. Each week (on days 0, 7, 14, 21, and 28), six mice from each group were sacrificed for analysis. To assess tumor proliferation, mice were injected i.p. with 5-bromo-2-deoxyuridine (BrdUrd; 100 mg/kg) 4 h before sacrifice. Blood was collected via cardiac puncture and plasma drug concentrations were measured by high-performance liquid chromatography as described previously (8). As i.p. ovarian xenografts disseminate throughout the peritoneal cavity, all macroscopic (visible) tumor nodules were excised, harvested, and weighed followed by fixation in 4% paraformaldehyde solution. Tumor tissues

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were paraffin embedded, processed, and sectioned. Markers of apoptosis [terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and caspase-3] were also examined.

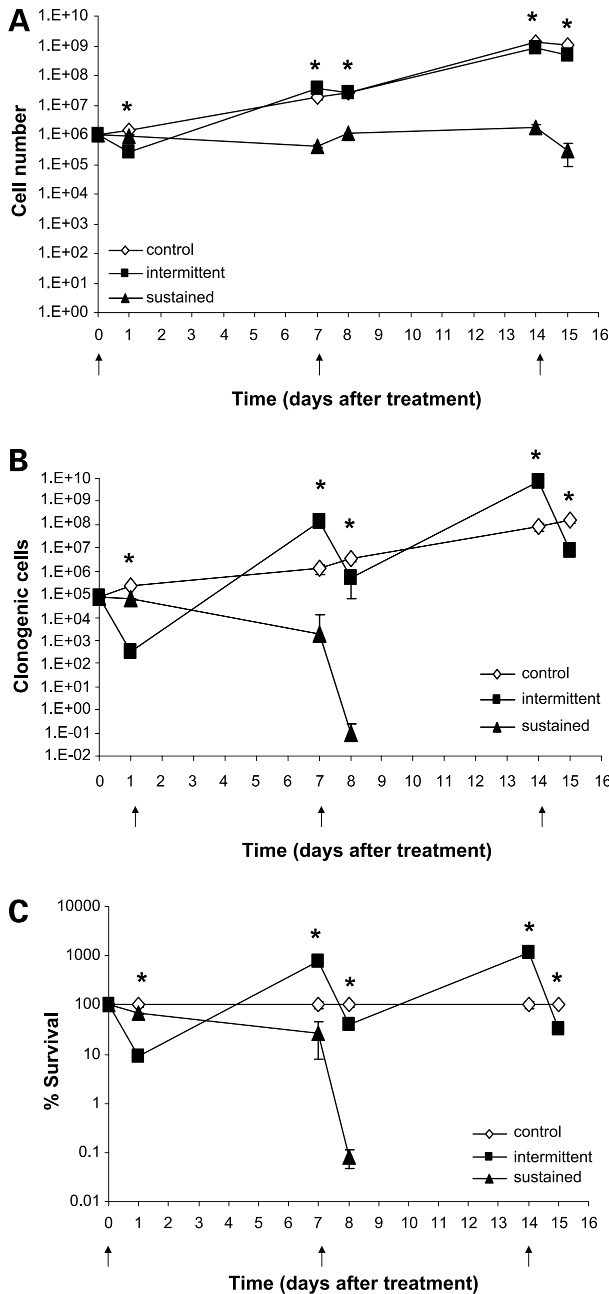

Figure 1. Effects of intermittent (4,000 nmol/L) and sustained (571 nmol/L) paclitaxel treatment on SKOV3 cells: (**A**) total cell number, (**B**) clonogenic cells, and (**C**) cell survival. *, $P < 0.05$, significant difference in total cell number, clonogenic cells, and cell survival between groups at all time points (ANOVA). Sustained treatment significantly reduced cell number and cell viability, whereas intermittent treatment significantly increased clonogenic cells in between treatment-free periods and with time, resulting in increased cell survival and tumor repopulation (ANOVA; Student's *t* test, $P < 0.05$). Mean ± SE. *Arrows,* intermittent 24-h paclitaxel treatment.

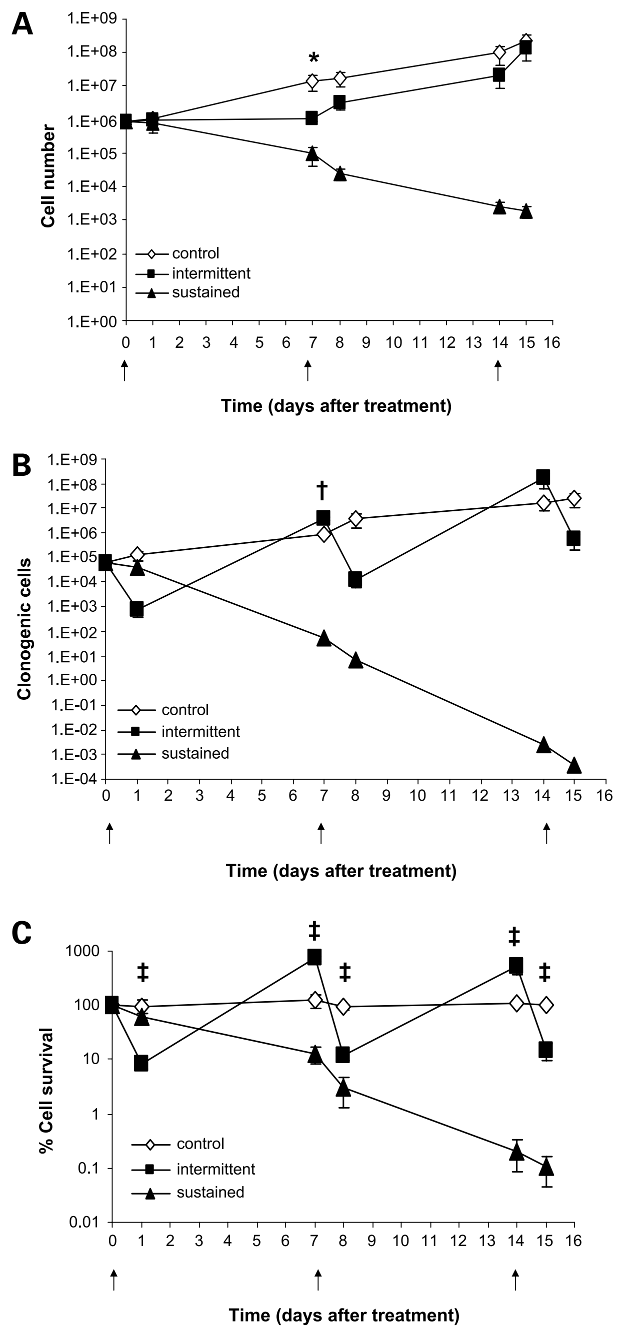

Figure 2. Effects of intermittent (4,000 nmol/L) and sustained (571 nmol/L) paclitaxel treatment on A2780 cells: (**A**) total cell number, (**B**) clonogenic cells, and (**C**) cell survival. *, $P < 0.05$, significant difference in the number of cells between the intermittent and the sustained groups (Student's *t* test). †, $P < 0.05$, significant difference in clonogenic cells between all groups (ANOVA) on day 7 only. ‡, $P < 0.05$, cell survival was significantly different between groups at all time points (ANOVA). Mean ± SE. *Arrows,* intermittent 24-h paclitaxel treatment.

Immunohistochemistry

Paraffin sections were dewaxed in xylene, passaged through graded alcohols, and rinsed in distilled water. Sections were then immersed in 10 mmol/L citrate buffer

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(pH 6) at 95°C to 100°C for 20 min in a microwavable pressure cooker. Sections were cooled and rinsed in PBS, following which they were treated with 1% pepsin (Sigma) in 0.01 N HCl (pH 2.0) for 15 min at 37°C. Endogenous peroxidase and biotin activity were blocked using 3% hydrogen peroxide and avidin/biotin blocking kit (Lab Vision). For the assessment of proliferation, sections were incubated at room temperature with the BrdUrd primary antibody (Invitrogen), 1:500 dilution for 1 h, followed with a biotinylated secondary antibody (Vector Laboratories) for 30 min and horseradish peroxidase–conjugated ultrastreptavidin labeling reagent (ID Labs) for 30 min. For the assessment of apoptosis, sections were incubated with active caspase-3 antibody (Chemicon), 1:50 dilution overnight, or with biotin-nucleotide cocktail and DNA polymerase 1 (Promega) for 1 h at 37°C for *in situ* TUNEL. Color development was done with freshly prepared NovaRed solution (Vector Laboratories). Sections were counterstained lightly with Mayer's hematoxylin, dehydrated in alcohols, cleared in xylene, and mounted in Permount (Fischer). Nuclei that stained brownish red were scored as positive and those that stained blue were scored as negative.

Quantification of Tumor Proliferation and Apoptosis

Tumor sections were imaged using a Nikon Coolpix 990 color camera mounted on a Nikon Eclipse e400 microscope. At least 10 fields (×400) were randomly selected from each slide. Positively and negatively stained nuclear areas were collected by using Image J analysis software (NIH). BrdUrd, caspase-3 and TUNEL labeling indices were determined as the ratio of areas occupied by the positively stained tumor cell nuclei to all tumor cell nuclei.

Statistical Analysis

Results are presented as mean ± SE. Data were analyzed using one-way ANOVA and the paired Student's t test of unequal variance for comparison between groups. Differences between groups were considered statistically significant at $P < 0.05$.

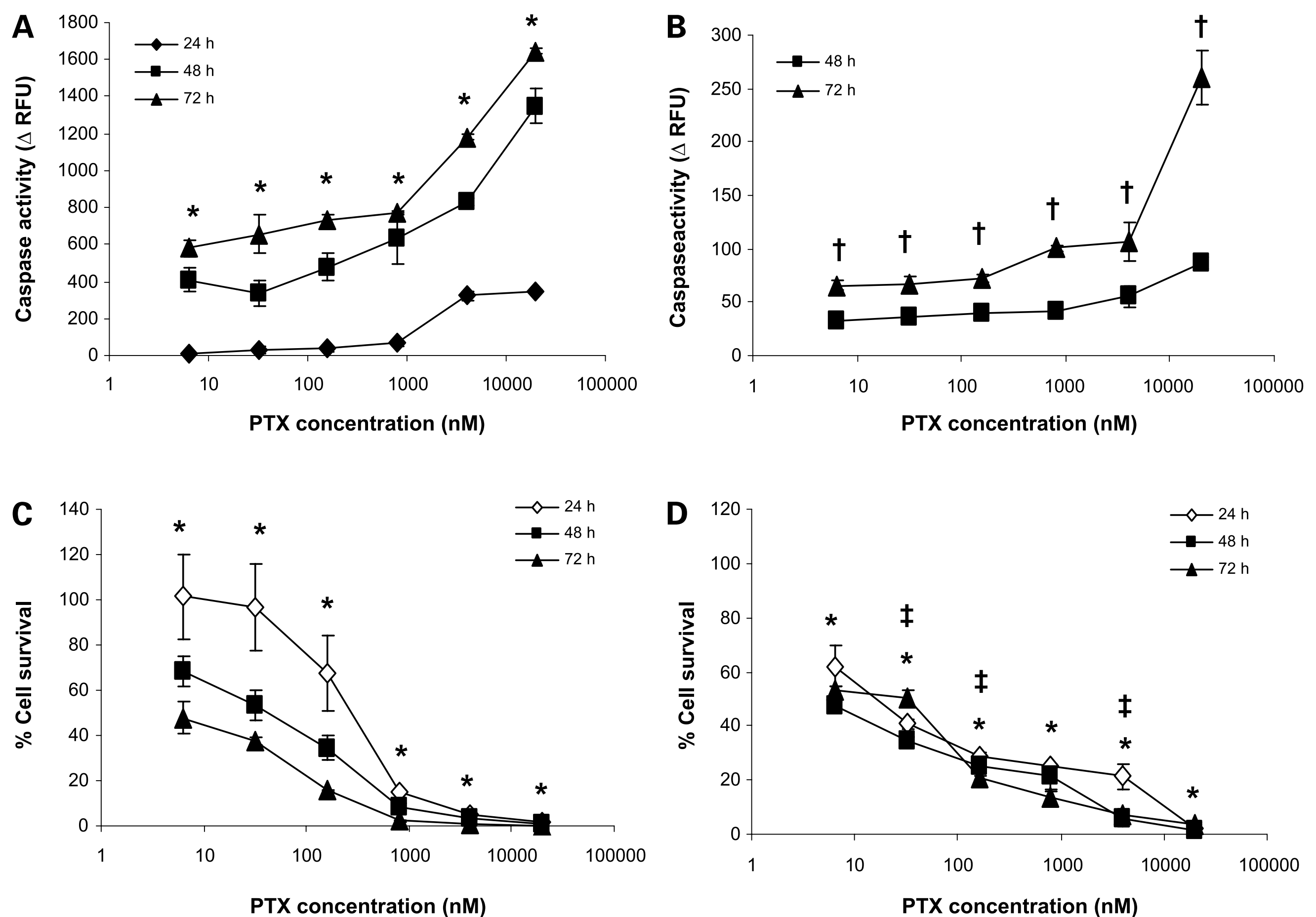

Figure 3. Effects of paclitaxel on caspase activity in SKOV3 (**A**) and A2780 (**B**) cells and on cell survival (**C** and **D**) at various concentrations and exposure times. *, $P < 0.05$ (ANOVA); †, $P < 0.05$ (Student's t test), caspase activity was augmented significantly with prolonging exposure and increasing concentration in both cell lines. Note that no caspase activity was observed in the A2780 cells following 24-h paclitaxel exposure at any concentration. *, $P < 0.05$, cell survival decreased significantly with increasing concentration in both cell lines (ANOVA). *, $P < 0.05$, increasing exposure time significantly decreased cell survival in SKOV3 cells (ANOVA). ‡, $P < 0.05$, in A2780 cells, these effects became significant at >32 nmol/L paclitaxel (ANOVA). Mean ± SE.

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Table 1. Effects of sustained and intermittent paclitaxel therapy on tumor growth and apoptosis in SKOV3 xenografts

Percentage		Days posttreatment initiation			
		0	7	14	21
Tumor weight inhibition	C →	0	0	0	0
	I →	0	43 ± 14	−81 ± 69	−16 ± 32
	S →	0	68 ± 19	98 ± 2*	81 ± 9*
Caspase-3	C →	0.4 ± 0.4	0.7 ± 0.5†	0.8 ± 0.7†	0.3 ± 0.3†
	I →	0.4 ± 0.4	0.6 ± 0.3†	2.0 ± 1.0†	0.2 ± 0.1†
	S →	0.4 ± 0.4	11 ± 2†	27 ± 5†	25 ± 11†
TUNEL	C →	0.2 ± 0.1	0†	3 ± 2†	3 ± 2†
	I →	0.2 ± 0.1	1.0 ± 0.5†	4 ± 2†	2 ± 1†
	S →	0.2 ± 0.1	28 ± 8†	86 ± 5†	62 ± 12†

Abbreviations: C, control, no treatment; I, intermittent therapy; S, sustained therapy.
*$P < 0.05$, sustained therapy resulted in significant tumor weight inhibition starting at day 14 posttreatment initiation in comparison with intermittent therapy (Student's t test).
†$P < 0.05$, significant difference in the degree of apoptosis between all groups (ANOVA). Apoptosis was highest in the sustained group and similar in the intermittent and control groups (Student's t test, $P < 0.05$). Caspase-3 and TUNEL indices correlated significantly in the sustained treatment group (regression analysis, $P < 0.05$).

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Results

Effects of Sustained and Intermittent Paclitaxel on Cell Growth and Clonogenic Survival

The effect of sustained versus intermittent paclitaxel dosing on tumor repopulation was investigated in the SKOV3 and A2780 human ovarian carcinoma cell lines. The growth of both cell lines was inhibited by paclitaxel (24-h exposure) in a concentration-dependent manner. Based on the survival curve obtained from clonogenic assays, 4,000 nmol/L paclitaxel was selected as the intermittent treatment concentration ($IC_{\sim 80\text{-}90}$) as similar cell kill was achieved in both cell lines and because clinically ~80% to 90% of tumor cell kill is accomplished with each treatment cycle. The concentration of the sustained treatment was calculated to be 571 nmol/L paclitaxel based on the $AUC_{intermittent}$. There was a significant difference in the PE, total cell number, surviving fraction, and the number of colony-forming SKOV3 cells between treatment groups (Fig. 1A-C). Sustained treatment significantly reduced the total number and the number of colony-forming SKOV3 cells, whereas intermitted treatment significantly increased the fraction of surviving tumor cells with time (Fig. 1A-C). Thus, treatment-free periods resulted in a significant gradual acceleration in the rate of SKOV3 repopulation (Fig. 1B). Similarly, total cell number, surviving fraction, and the number of A2780 clonogenic cells were also significantly inhibited by sustained paclitaxel treatment, whereas intermittent treatment resulted in an increase in A2780 survival with time and in between treatment cycles (Fig. 2A-C). Intermittent treatment resulted in significantly greater clonogenic cell survival (up to ~50-fold higher) in SKOV3 cells than A2780 cells, whereas sustained treatment had similar effects in both cell lines.

Effects of Increased Paclitaxel Exposure on Apoptosis and Survival

Because apoptosis is the major form of cell death in response to paclitaxel and is an indicator of cytotoxicity, we investigated the degree of caspase activation at various paclitaxel concentrations and exposure times in the SKOV3 and A2780 human ovarian cancer cell lines. There was a significant elevation in caspase activation with an increase in concentration and exposure time in the SKOV3 cells (Fig. 3A). Although caspase induction was not evident following a 24-h exposure to paclitaxel in A2780 cells, an induction in caspase activity was observed at 48 h and was significantly elevated with increasing concentration and exposure time (Fig. 3B). Cell survival was decreased in a concentration- and time-dependent fashion in both cell lines and had a significant inverse correlation with caspase activity (Fig. 3C and D).

Effects of Sustained and Intermittent Paclitaxel Therapy on Tumor Growth and Repopulation in SKOV3 Xenografts

The 3-week treatment with sustained paclitaxel administration resulted in significant growth reduction of tumors (up to 98% tumor weight inhibition), whereas intermittent therapy had no effect (Table 1; Fig. 4A). Furthermore, there was a significant difference in the proliferation index of tumors between groups as indicated by the incorporation of BrdUrd (Fig. 4B). Two to 3 weeks of treatment with sustained paclitaxel administration significantly reduced the proliferation index of tumors, whereas intermittent therapy resulted in a significant gradual increase in tumor proliferation over the same period, indicating acceleration in tumor repopulation with time (Figs. 4B and 5). Furthermore, this increase in proliferation significantly correlated with tumor weight.

Effects of Sustained and Intermittent Therapy on Apoptosis in SKOV3 Xenografts

To further characterize tumor response to treatment, we investigated the degree of apoptosis as measured by caspase-3 and TUNEL. Two to 3 weeks of treatment with sustained administration significantly increased the proportion of apoptotic tumor cells, whereas intermittent

therapy had no effect (Table 1; Fig. 5). Moreover, there was a significant correlation between capsase-3 and TUNEL indices in the sustained group.

Paclitaxel Concentration Levels in Plasma and Liver

Paclitaxel concentration levels were measured in plasma and liver each week. As these levels were measured in samples taken 1 week following treatment, it was not surprising that paclitaxel levels were below detection limits in the intermittently dosed group. Paclitaxel plasma concentration levels from the sustained therapy group were 43 ± 5, 55 ± 4, and 52 ± 4 ng/mL on days 7, 14, and 21 posttreatment initiation, respectively. Paclitaxel liver levels were 16 ± 6, 12 ± 1, and 24 ± 12 μg/g on days 7, 14, and 21 posttreatment initiation, respectively. Hence, the Alzet pump released paclitaxel in a sustained manner achieving the expected C_{ss} rates for the administered dose.

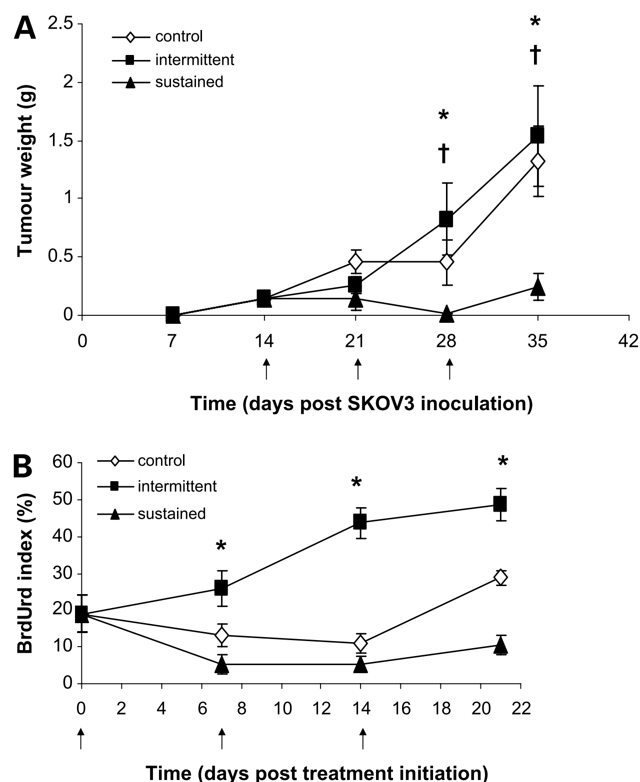

Figure 4. Tumor growth and proliferation of SKOV3 xenografts after intermittent and sustained paclitaxel therapy. **A,** tumor growth curves. **B,** BrdUrd tumor proliferation indices. Paclitaxel therapy (60 mg/kg total over 3 wk) was initiated 14 d after SKOV3 inoculation with mean tumor weight of 0.14 ± 0.05 g. Intermittent therapy consisted of 20 mg/kg on q7d × 3 schedule and sustained therapy consisted of surgical implantation of Alzet pumps releasing 20 mg/kg/wk. *, $P < 0.05$ (Student's *t* test); †, $P < 0.05$ (Student's *t* test), a significant reduction in tumor growth in the sustained therapy group at 14 and 21 d posttreatment initiation in comparison with nontreated controls (*) and the intermittent therapy group (†). *, $P < 0.05$, proliferation, as assessed by BrdUrd indices varied significantly among groups (ANOVA) and was significantly higher and increased with time in tumors obtained from the intermittent treatment group. $P = 0.05$, tumor proliferation significantly correlated with tumor growth in the intermittent group as assessed by regression analysis. Mean ± SE. Arrows, intermittent paclitaxel treatment.

Discussion

Tumor repopulation remains a neglected factor in the treatment failure of ovarian cancer. We show that sustained paclitaxel therapy diminishes the rates of ovarian tumor repopulation and that intermittent therapy results in accelerated rates of tumor repopulation in between treatment cycles and thus limits efficacy.

Our *in vitro* findings showed that treatment-free intervals between intermittent cycles of paclitaxel chemotherapy result in significantly increased proliferation and fraction of surviving clonogenic tumor cells. In contrast, sustained exposure augmented the cytotoxic effect of paclitaxel and significantly decreased clonogenic survival. The effects of intermittent treatment on tumor repopulation were more profound in the SKOV3 cells as the number of clonogenic cells increased with each successive treatment-free interval and significantly exceeded that of controls and sustained treatment groups. Compared with sustained exposure, intermittent paclitaxel treatment of A2780 cells also increased cell repopulation but was not different from untreated controls. Controls from both cell lines had similar PE and clonogenic cell number throughout the study. However, a 14- to 47-fold greater number of clonogenic cells was observed with SKOV3 cells compared with A2780 cells after intermittent treatments. This suggests that paclitaxel promotes more rapid and aggressive tumor repopulation in SKOV3 cells. This difference in treatment response may be due to the distinct genetic and pathologic profiles of the cell lines in addition to prior drug exposure. The SKOV3 cell line is obtained from a previously treated patient, whereas the A2780 cell line is attained from an untreated patient. There is evidence that regrowth of tumors is faster after a single dose of radiation than growth of nonirradiated control tumors and that it accelerates with consecutive treatments (11–13). It is likely that this may also be witnessed with chemotherapy with previous drug exposure playing a role in repopulation acceleration. Therefore, it is plausible that more aggressive repopulation of A2780 cells might be observed with subsequent treatment cycles.

Similarly, *in vivo*, in the SKOV3 xenograft model, intermittent paclitaxel chemotherapy resulted in significantly augmented tumor cell repopulation with each successive treatment cycle, whereas sustained paclitaxel chemotherapy had the opposite effect. Compared with nontreated controls, BrdUrd uptake was 2- to 4-fold greater in tumors obtained from the intermittent group after two to three treatment cycles and 5- to 8-fold greater than that observed in the sustained treatment group. These differences remained significant throughout the course of treatment and until the termination of the study. Furthermore, tumor response, as measured by total tumor weight, in the sustained group was 68% to 98%, whereas in the intermittent group it decreased from 43% in the first week to absolutely none. Tumor burden in the intermittent group was up to 2-fold greater than controls and up to 88-fold greater than the sustained group and was found to be significantly correlated with BrdUrd uptake. Indeed, clinical studies have shown that administration of paclitaxel

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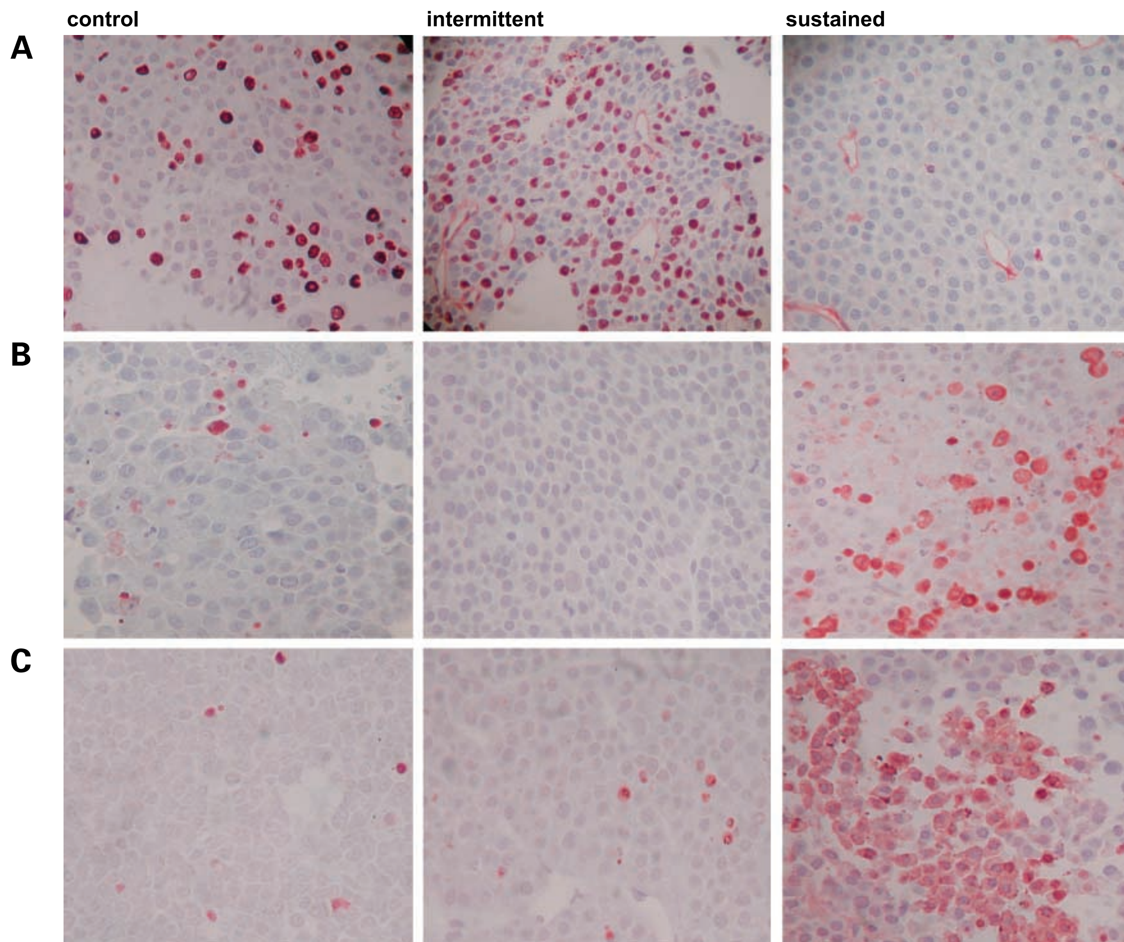

Figure 5. Representative tumor sections displaying the effect of intermittent and sustained paclitaxel therapy on tumor proliferation and apoptosis in SKOV3 xenografts. **A,** BrdUrd. **B,** Caspase-3. **C,** TUNEL. Paraffin sections (5 μm) at ×400 magnification. Nuclei stained red/brown were scored as positive for BrdUrd, caspase-3, or TUNEL and nuclei that stained blue were scored as negative.

weekly, rather than the standard every 3-week regimens, has minimal toxicity and greater efficacy in breast cancer patients (14–16). In this context, sustained administration of paclitaxel may even achieve greater efficacy through continuous cytotoxic exposure. This approach to treatment may inhibit tumor repopulation between cycles and limit the emergence of malignant cell populations resistant to chemotherapy.

Induction of apoptosis in cancer cells is a crucial mechanism of chemotherapeutic action and a measurement of apoptotic response may be applied in the determination of chemosensitivity. Our studies show extensive apoptosis and enhanced tumor responsiveness with sustained paclitaxel exposure. Paclitaxel induced caspase activation in a concentration- and time-dependent manner in both SKOV3 and A2780 cells. Interestingly, paclitaxel induced more extensive and immediate caspase activation in SKOV3 cells in comparison with A2780 cells. This difference in caspase activation between the two cell lines may be attributed to their distinct molecular and genetic profiles. For instance, the SKOV3 cell line is p53 deficient, whereas the A2780 cell line expresses wild-type p53 (17). p53 status has an effect on apoptosis progression; it has been found that paclitaxel is more cytotoxic to p53-mutant/deficient cells than wild-type p53 tumor cells (18).

Although there was a difference in the degree of caspase activation between the SKOV3 and A2780 cells, increasing concentration and duration of paclitaxel exposure significantly augmented caspase induction and significantly decreased clonogenic survival in both cell lines. At low paclitaxel concentrations, cell survival was not significantly altered, whereas increasing exposure time without altering concentration enhanced cytotoxicity. Indeed, greater efficacy has been observed with longer treatment durations; increasing exposure time has been reported to result in reduced clonogenic survival, greater G_2-M block and lower PE in human ovarian, breast, lung, cervical, colorectal, and pancreatic cancer cells (19–26).

Likewise, *in vivo*, tumor apoptosis was significantly greater in the sustained treatment group, whereas it was very low and similar in both the control and the intermittent groups. For instance, in the sustained group, caspase-3

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and TUNEL indices were up to 37- and 123-fold higher, respectively, than in the intermittent group. Intermittent chemotherapy offers inadequate drug penetration to tumor cells as concentrations achieved by this treatment method are short-lived and limited to the periphery of the tumor. In this context, remaining tumor cells that are not killed repopulate the tumor during treatment-free intervals, resulting in poor efficacy. The Alzet pumps provided continuous release of paclitaxel directly at the tumor site, allowing for enhanced drug penetration into the inner layers of the tumor and greater tumor cell kill. Further targeting of vascularized tumors was achieved through the circulation as sustained paclitaxel plasma levels of 50 to 64 nmol/L (43-55 ng/mL) were achieved, which are clinically relevant but are below toxicity levels. Paclitaxel was below detection limits in the intermittent group. Peak plasma levels following a single i.p. bolus paclitaxel administration (20 mg/kg) have been reported to be >95,000 ng/mL, which exceed toxic levels. We detected paclitaxel liver concentrations of 12 to 24 μg/g in the sustained treatment group, whereas concentrations of 50 to 60 μg/g have been reported following a single i.p. bolus dose of 20 mg/kg. Therefore, intermittent therapy offers high systemic toxicity and a short-lived therapeutic window. Consequently, it is plausible that inadequate amounts of drug reach the tumor mass and could be limited to the periphery. Indeed, improved and more even tumor drug penetration has been shown with increased exposure time to paclitaxel both in xenografts and in patient tumors (27). It is possible that escalating doses could increase tumor drug concentrations; however, the administration of high dosages in the clinical setting is limited by toxicity. Therefore, treatment-free intervals between cycles of chemotherapy are required to allow normal tissues to regenerate; however, during these treatment-free intervals, remaining tumor cells also proliferate, resulting in treatment failure. As chemotherapeutics are generally administered i.v., limited tumor drug penetration via the systemic vasculature is a critical obstacle in treatment efficacy.

In summary, because the majority of epithelial ovarian primary and metastatic tumors remain within the peritoneal cavity, strategies such as localized and sustained chemotherapy may have the potential to improve the outcome of chemotherapy. Localized administration can provide high regional drug concentrations directly at the tumor site while limiting systemic toxicity, whereas sustained regimens may overcome tumor repopulation during treatment-free periods. This strategy may be the ideal scenario for the treatment of residual disease postoperatively and ultimately in the prevention of recurrence. Optimal dosing intervals for the treatment of ovarian cancer still remain to be determined.

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