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ORIGINAL ARTICLE

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Retention of paclitaxel in cancer cells for 1 week in vivo and in vitro

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Abstract *Purpose*: Clinically, the administration of paclitaxel for ovarian cancer on a dose-dense weekly schedule, rather than the conventional every-3-week schedule, might demonstrate greater tumor-cell death. Here, we investigate the pharmacokinetics and the pharmacodynamics of weekly paclitaxel in cancer cells in vivo and in vitro. *Experimental design*: Paclitaxel concentrations were measured by HPLC, and apoptotic cells were detected by TUNEL assay in paclitaxel-pretreated cervical cancer cells treated with paclitaxel (10 ng/ml) and in the tissues of cervical cancer patients treated with weekly paclitaxel (60 mg/m^2/week). Polymerized tubulin was detected with a tubulin polymerization assay, and the BrdU cell proliferation assay was used to assess the effect of paclitaxel. *Results*: Paclitaxel remained in the cancer tissues of six patients for 6 days after the last medication. In vitro, paclitaxel was retained in all cell lines for 24 h after its removal from the medium, and paclitaxel was still detectable in CaSki cells on day 7. Simultaneous treatment with depolymerizing drugs inhibited the retention of paclitaxel in cells and paclitaxel-induced polymerization of tubulin. After paclitaxel treatment, apoptotic cells were detected in cancer tissues and CaSki cells for 1 week. Under high magnification, apoptotic cells on day 7 after paclitaxel treatment showed multinucleation. *Conclusions*: Paclitaxel is unusual in that it accumulates especially in cancer cells and induces apoptosis for 1 week in vivo and in vitro. On the other hand, paclitaxel could not be detected in cancer tissues after 2 weeks. The administration of paclitaxel on a weekly schedule, rather than the standard every-3-week schedule, might produce greater tumor-cell death.

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Introduction

Combination chemotherapy consisting of paclitaxel and carboplatin has become the standard first-line treatment regimen for patients with advanced ovarian cancer. Meanwhile, paclitaxel is recognized as one of the most active cytotoxic agents in the treatment of ovarian cancer, breast cancer, lung cancer and other gynecologic cancers. For example, paclitaxel is being investigated as an active agent in cervical cancer [22].

In one previously reported trial, a weekly paclitaxel regimen produced objective tumor regressions in patients with ovarian cancer previously treated with paclitaxel on an every-3-week program [16]. More recently, Green et al. [6] reported that patients with breast cancer receiving a weekly paclitaxel regimen had a higher pathologic complete response rate than patients treated with paclitaxel on an every-3-week program, with improved breast cancer conservation rates. The administration of paclitaxel on a weekly schedule, rather than the standard every-3-week schedule, might produce greater tumor-cell death.

Paclitaxel binds to β-tubulin and stabilizes microtubules, repressing the dynamic instability of spindle microtubules, and this results in a cell cycle block at the metaphase-to-anaphase transition [7]. This arrest in mitosis correlates with paclitaxel-induced cytotoxicity [30]. Paclitaxel-induced apoptosis has been associated with two different mechanisms of cell-cycle arrest [29, 30]. In one mechanism, arrest in prometaphase is suggested to trigger rapid cell death independent of p53 [30]. In the other mechanism, which is p53-dependent, paclitaxel at concentrations less than that required for a G2-M block induces the formation of multipolar spindles and an aneuploid G1 population of cells [3]. The aneuploid

G1 cells may result from aberrant mitosis and die slowly of apoptosis [30].

Materials and methods

Cell culture and reagents

HeLa cells and CaSki cells, which are both human uterine cervical cancer cell lines, were gifts from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). CaSki cells were maintained in RPMI 1640 with L-glutamine (Nacalai Tesque, Kyoto, Japan), supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA). HeLa cells were maintained in Eagle's MEM (Nacalai Tesque) with nonessential amino acids, sodium pyruvate and 10% FBS. NCC16-P11 cells, which are normal human cervical cells, were purchased from the Health Science Research Resources Bank (Osaka, Japan) and were maintained in MCDB153 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 μg/ml insulin (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 10 μg/ml transferrin (Sigma-Aldrich), 0.1 mM phosphorylethanolamine (Sigma-Aldrich), 0.1 mM ethanolamine (Sigma-Aldrich), 10 ng/ml EGF (Sigma-Aldrich) and 40 μg/ml bovine pituitary extract (BD Biosciences, Beford, MA, USA).

Paclitaxel and carboplatin were gifts from Bristol-Myers Squibb Company (Princeton, NJ, USA). Vinblastine, colchicine and nocodazole were purchased from Sigma-Aldrich. Anti-β-tubulin monoclonal antibody was purchased from BD Biosciences.

Patients and samples

Six patients (from Pt. 1–6) who had been diagnosed with uterine cervical cancer (FIGO stage II) were treated with weekly paclitaxel (60 mg/m^2/week) as a neoadjuvant chemotherapy (NAC) for 2–4 weeks and then underwent radical hysterectomy 6 days after the final paclitaxel treatment. Two patients (Pt. 7 and Pt. 8) who had been diagnosed with uterine cervical cancer (FIGO stage II) were treated with weekly paclitaxel (60 mg/m^2/week) as a NAC for 4 weeks and then underwent radical hysterectomy 14 days after the final paclitaxel treatment. One patient (Pt. 9) who had been diagnosed with uterine cervical cancer (FIGO stage Ib) underwent radical hysterectomy without NAC. A small amount of cancer tissue from each patient was stored at (80°C for measurement of paclitaxel concentration in tissues and then all specimens were fixed with 10% formalin and embedded in paraffin wax for histological examination. Heparinized blood samples were obtained at the end of paclitaxel infusion and at 24 h after completion of infusion. Informed consent was obtained from all patients before surgery and examination of the specimens used in this study.

Measurement of paclitaxel and carboplatin concentration

Cells were grown to 90% confluency in 150-mm dishes and treated with dimethyl sulfoxide (DMSO) or 10 ng/ml paclitaxel alone or simultaneously with paclitaxel and either 100 nM vinblastine, 1 μM colchicine, or 10 μM nocodazole for 16 h. Then the medium was changed and the cells were maintained for an additional 24 h in normal medium. After that, the cells were collected by centrifugation and the cell pellets were stored at −80°C until required for measurement of paclitaxel concentration. In the time-course study, cells were grown to 90% confluency in 150-mm dishes, treated with DMSO or 10 ng/ml paclitaxel for 16 h, and then incubated with normal medium. The medium was changed every other day, cells were collected by centrifugation on days 1, 3, 5 and 7, and the cell pellets were stored at −80°C.

Paclitaxel concentrations in cells and tissues were determined by high-performance liquid chromatography (HPLC) according to the method of Huizing et al. [8] with modifications developed by SBS Inc. (Kanagawa, Japan). Briefly, the HPLC system consisted of a LC-9A chromatograph system (Shimadzu, Ktoto, Japan), SPD-6AV UV detector at 227 nm, and chromatopac C-R4A data-processor. Plasma samples (0.5 ml) diluted with 2 ml water were applied to a solid-phase extraction column, Scp-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA), and conditioned with 5 ml acetonitrile followed by 10 ml water. After evaporation to dryness, the residue was reconstituted in 200 μl 45% acetonitrile−55% 2 mM H_3PO_4, and 100 μl of reconstituted sample was injected onto the HPLC column. The paclitaxel concentration was quantitated by linear regression analysis of the peak height ration (paclitaxel:*n*-hexyl *p*-hydroxy benzoate) versus the standard curve generated from the solution of paclitaxel with *n*-hexyl *p*-hydroxy benzoate diluted with acetonitrile−2 mM H_3PO_4 (45:55). Data were expressed as nanograms per milliliters. After measurement of weight, cell and tissue samples were homogenized in 2 ml water and then were processed in a similar manner. Data were expressed as nanograms per gram wet.

The cell lines were treated with 10 ng/ml carboplatin for 16 h and then incubated with normal medium for an additional 24 h. Platinum concentrations in cells were determined by flameless atomic absorption spectrometry according to the method of Pera et al. [21] with modification developed by SBS Inc. Briefly, cell and tissue samples were weighted and triturated to yield homogeneous samples. Each sample was liquefied and digested by heating with 60% HNO_3. When digest was almost dry, the residue was taken up in 0.1 M HCl, evaporated, and resolubilized in a known volume of the HCl. Samples were atomized flamelessly at approximately 2700° in GTA-100 flameless atomizer (Varian Technologies Japan, Ltd., Tokyo, Japan). Absorbance was measured at 265.9 nm with Spectro AA 880 Zeeman atomic absorption spectrophotometer (Varian Technologies Japan, Ltd.). Data were expressed as nanograms per gram wet.

Tubulin polymerization assay

Tubulin polymerization was quantified with a simple assay based on that of Minotti et al. [19]. CaSki cells grown to 80% confluency in 100-mm dishes were treated with DMSO or 10 ng/ml paclitaxel alone or simultaneously with paclitaxel and either 100 nM vinblastine, 1 μM colchicine, or 10 μM nocodazole for 16 h. Then the medium was changed and cells were maintained for an additional 24 h in normal medium. The cells were washed twice with phosphate buffered saline (PBS), lysed for 5 min with 300 μl of hypotonic buffer [1 mM $MgCl_2$, 2 mM EGTA (Sigma-Aldrich), 0.5% NP40 (Nacalai Tesque), 2 mM phenylsulfonyl fluoride (Nacalai Tesque), 200 U/ml aprotinin (Calbiochem, San Diego, CA, USA), 100 μg/ml soybean trypsin inhibitor (Sigma-Aldrich), 5 mM ε-aminocaproic acid (Sigma-Aldrich), 0.01 nM benzamidine (Nacalai Tesque) and 20 mM Tris–HCl, pH 6.8] and transferred to Eppendorf tubes. The wells were rinsed with an additional 300 μl of hypotonic buffer and this was combined with the lysates. The samples were centrifuged at 12,000*g* for 10 min at room temperature, the 600 μl of supernatants containing soluble (cytosolic) tubulin were removed, and the pellets, containing polymerized tubulin, were resuspended in 600 μl of hypotonic buffer. Samples of each fraction (15 μl) were mixed with 5 μl of 4×SDS-PAGE buffer (45% glycerol, 20% β-mercaptoethanol, 9.2% SDS, 0.04% bromphenol blue, and 0.3 M Tris–HCl, pH 6.8), heated at 95°C for 5 min, and analyzed by SDS-PAGE on a 12.5% polyacrylamide gel. Immunoblotting was performed with 1.0 μg/ml of anti-β-tubulin antibody and a dilution of 1:20,000 donkey anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA, USA) and the membrane was stained with 3,3′-diaminobenzidine (DAB) for 10 min.

BrdU cell proliferation assay

CaSki cells (100 cells per well) were seeded into a 96-well plate and then incubated for 24 h. The cells were treated with DMSO or 10 ng/ml paclitaxel for 16 h, washed twice with PBS and incubated with normal medium. On day 1 or day 7 after removal of the DMSO or paclitaxel, cells were incubated with 5-bromo-2′-deoxyuridine (BrdU) for 24 h, and then the uptake of BrdU was measured according to the manufacturer's protocol (Calbiochem). The absorbance in each well was measured with a spectrophotometric plate reader at 450 and 595 nm and the results calculated as A_{450}–A_{595}.

TUNEL assay

Paclitaxel-induced apoptosis was assayed by the terminal deoxynucleotidyl transferase (TdT)-mediated incorporation of biotinylated nucleotides. The TUNEL assay was performed with the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, CaSki cells (1×10^5 cells and 1×10^4 cells) were plated on poly-D-lysine-coated 2-well culture slides (Becton Dickinson, Bedford, MA, USA) and after incubation for 24 h, 10 ng/ml paclitaxel was added to the culture medium. Following incubation with paclitaxel for 16 h, the medium was replaced with normal medium until day 1 (1×10^5 cells) and day 7 (1×10^4 cells). Cells were fixed with 4% paraformaldehyde for 25 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Cells were then incubated with TdT reaction mixture containing biotinylated nucleotide and recombinant TdT enzyme (100 μl) at 37°C for 60 min. To ensure homogeneous distribution of the TdT reaction mix and to avoid evaporative loss, slides were covered with a cover slide during incubation. To terminate the reactions, the slides were incubated with sodium chloride-sodium citrate buffer (SSC) for 15 min at room temperature. After rinsing the slides, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide for 5 min, and then the slides were incubated with Horseradish peroxidase-labeled streptavidin (streptavidin HRT) solution for 30 min at room temperature and rinsed twice with PBS. Then 50 μl of DAB solution was added and the slides were incubated for 5 min, rinsed with water, and counterstained with hematoxylin.

Paraffin-embedded tissue blocks were cut into 5-μm-thick sections and mounted on glass slides. The sections were kept at 4°C overnight, deparaffinized in xylene, dehydrated and treated with proteinase-K for 10 min at room temperature. The slides were then rinsed twice with PBS and the TUNEL assay was performed as described earlier. The nuclei of apoptotic cells were stained with DAB, while all nuclei were stained with hematoxylin.

Statistical analysis

Data were expressed as the mean ± SD. Differences between groups were tested for statistical significance using Student's *t* test. $P<0.05$ denotes a statistically significant difference.

Results

Paclitaxel concentration in cancer tissues

The concentrations of paclitaxel in the cancer tissues and blood samples are shown in Table 1. Paclitaxel was retained in the cancer tissues during those 6 days, whereas paclitaxel could not be detected in the cancer tissues of patients without chemotherapy and who underwent chemotherapy 14 days earlier. Paclitaxel plasma concentrations were 590 (439–882) ng/ml at the end of paclitaxel infusion (Table 1) and 10 ng/ml (lower limit of detection) at 24 h after completion of infusion (data not shown).

Table 1 Concentration of paclitaxel in uterine cervical cancer tissues and blood samples

Patient no.	Tissues (ng/g wet)	Blood samples (ng/ml)
Pt. 1 (6 days)	521	
Pt. 2 (6 days)	384	
Pt. 3 (6 days)	296	448
Pt. 4 (6 days)	163	
Pt. 5 (6 days)	377	882
Pt. 6 (6 days)	353	439
Pt. 7 (14 days)	0	
Pt. 8 (14 days)	0	
Pt. 9 (no drug)	0	

Paclitaxel remains in cells for up to 1 week

To confirm that paclitaxel remains in cells for such a long time, CaSki cells, HeLa cells and NCC16-P11 cells (normal human cervical cells) were tested in culture. The growth curves for these cell lines are shown in Fig. 1. The doubling time of CaSki cells, HeLa cells and NCC16-P11 cells were 1.6, 0.9 and 5.7 days, respectively. Paclitaxel was retained in all the cell lines for 24 h after the removal of paclitaxel (Fig. 2a). The retention of cellular paclitaxel did not depend on cell growth, and normal cells retained paclitaxel at only relatively low concentrations. In contrast, when the cell lines were treated with 10 ng/ml carboplatin for 16 h and then incubated with normal medium for an additional 24 h, carboplatin could not be detected in the cells (data not shown). This indicates that the retention of anti-tumor drugs in cells is characteristic of paclitaxel.

To assess how long paclitaxel is retained in cells, paclitaxel concentrations in CaSki cells were measured 1, 3, 5 and 7 days after the removal of paclitaxel. Paclitaxel was detected at a concentration of 1,880±203 ng/g wet on day 1 and then gradually decreased, but on day 7 paclitaxel was still retained in the cells at a concentration of 432±185 ng/g wet (Fig. 2b).

Effect of microtubule-depolymerizing drugs on paclitaxel retention and paclitaxel-induced tubulin polymerization

Paclitaxel interacts with polymerized tubulin and prevents depolymerization. Paclitaxel and depolymerizing drugs (vinblastine, colchicine, or nocodazole) bind differently to tubulin and have opposing mechanisms of action. To investigate how paclitaxel was retained in cells, CaSki cells were treated with DMSO or 10 ng/ml paclitaxel alone or simultaneously with paclitaxel and depolymerizing drugs. When cells were treated with paclitaxel alone, paclitaxel was retained in cells to the extent of 1,983±154 ng/g wet weight 24 h after the removal of the drug, but the retention of paclitaxel could be inhibited by simultaneous treatment with a depolymerizing drug (vinblastine, colchicine, or nocodazole) (Fig. 2c). A combination of 10 ng/ml paclitaxel and 100 nM vinblastine yielded a paclitaxel concentration of 186 ng/g wet, only 9.6% of the concentration observed for paclitaxel alone, while the other two microtubule-depolymerizing drugs completely inhibited paclitaxel retention.

Using a tubulin polymerization assay, we confirmed that paclitaxel induced tubulin polymerization and that depolymerizing drugs inhibited paclitaxel-induced tubulin polymerization 24 h after removal of paclitaxel (Fig. 2d). Treatment with 100 nM vinblastine sharply reduced paclitaxel-induced polymerized tubulin, although a faint band of polymerized tubulin could still be seen. The other depolymerizing drugs, colchicines and nocodazole, completely inhibited paclitaxel-induced tubulin polymerization. The extent of paclitaxel-induced tubulin polymerization was consistent with the concentration of paclitaxel remaining in cells after 24 h.

Inhibition of cell growth by paclitaxel retention

To investigate the effect of paclitaxel retention for 1 week, a BrdU cell proliferation assay was performed. Because only a few cells were seeded, there was no significant difference in BrdU uptake between DMSO and paclitaxel treatment on day 1 (Fig. 3). However, by day 7, paclitaxel treatment had significantly suppressed cell proliferation ($P < 0.05$).

Paclitaxel-induced apoptosis at day 7

Apoptosis is the main cause of the cytotoxicity induced by paclitaxel [18]. To investigate the mechanism of inhibition of cell proliferation by paclitaxel at day 7, we used the TUNEL method to detect apoptotic cells. CaSki cells were treated with DMSO or paclitaxel for 16 h in 2-well culture slides. After removal of the paclitaxel, apoptotic cells were detected with DAB at days 1 and 7 (Fig. 4). In

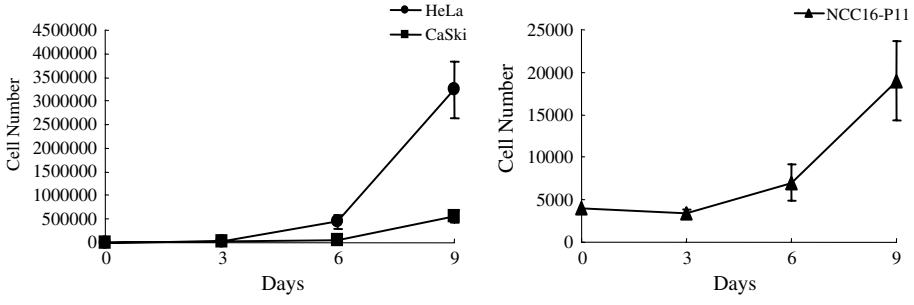

Fig. 1 Growth of uterine cervical cell lines HeLa cells (*circles*), CaSki cells (*squares*) and NCC16-P11 cells (*triangles*) were seeded and the cells were counted on days 3, 6 and 9. Data were expressed as the mean ± SD

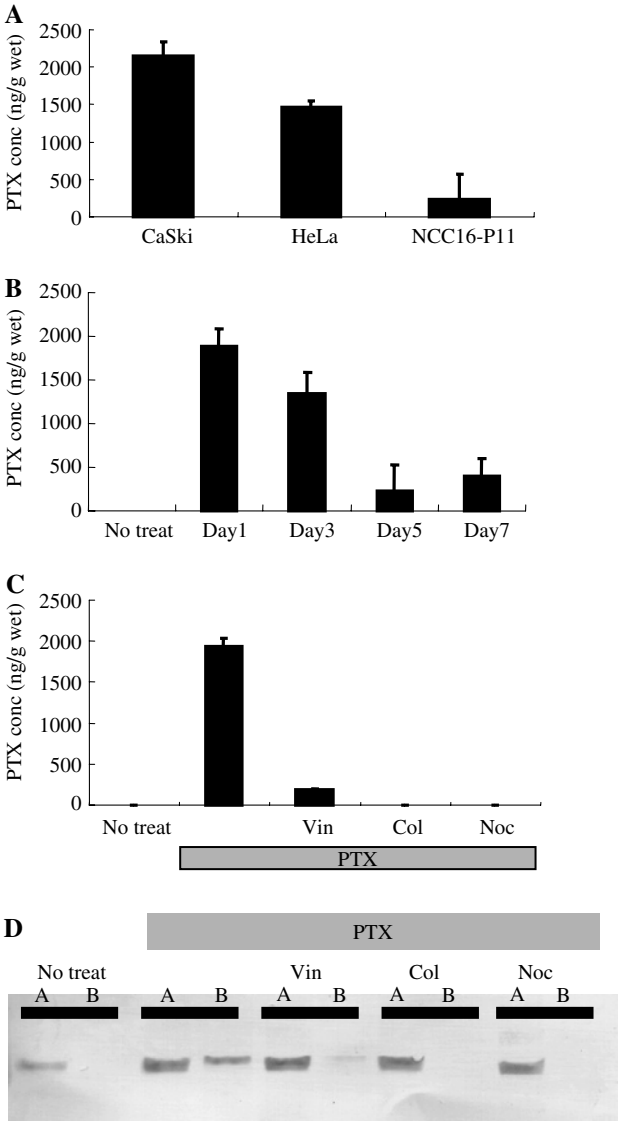

Fig. 2 **a** Paclitaxel concentration in different cell lines. CaSki, HeLa and NCC16-P11 cells were treated with dimethyl sulfoxide (DMSO) or 10 ng/ml paclitaxel for 16 h. Afterwards the medium was changed and the cells were maintained for an additional 24 h in normal medium. Cells were then collected and paclitaxel concentrations were determined. Data were expressed as the mean ± SD. **b** Time-course of paclitaxel concentration. CaSki cells were treated with DMSO or 10 ng/ml paclitaxel for 16 h. Then the medium was changed and cells were maintained in normal medium. On days 1, 3, 5 and 7, cells were collected and paclitaxel concentrations were determined. Data were expressed as the mean ± SD. **c** Effect of depolymerizing drugs on retention of paclitaxel in cells. CaSki cells were treated with DMSO or 10 ng/ml paclitaxel (PTX) alone or simultaneously with paclitaxel and either 100 nM vinblastine (Vin), 1 μM colchicine (Col), or 10 μM nocodazole (Noc) for 16 h. Then the medium was changed and the cells were maintained for an additional 24 h in normal medium. The cells were collected and paclitaxel concentrations were determined. Data were expressed as the mean ± SD. **d** Tubulin polymerization assay. Caski cells were treated with DMSO or 10 ng/ml paclitaxel (PTX) alone or simultaneously with paclitaxel and either 100 nM vinblastine (Vin), 1 μM colchicine (Col), or 10 μM nocodazole (Noc) for 16 h. Then the medium was changed and the cells were maintained for an additional 24 h in normal medium. The cells were lysed with hypotonic buffer, and the supernatants containing soluble tubulin (A) and the pellets containing polymerized tubulin (B) were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel. Immunoblotting was performed with anti-β-tubulin antibody and the membrane was developed with DAB

this study, TUNEL staining was restricted to the nuclei of apoptotic cells. On day 1, many cells had been removed by paclitaxel treatment and also many apoptotic cells were detected with the DAB stain (Fig. 4b). On day 7, some apoptotic cells were still detected with the DAB stain, while the growth of cells, which were not affected by paclitaxel, was also observed (Fig. 4d). By day 14 there was no difference in cell growth between DMSO and paclitaxel treatment (data not shown). Under high magnification, apoptotic cells on day 7 after paclitaxel treatment showed multinucleation (Fig. 4e).

Effect of weekly paclitaxel followed by operation

After paclitaxel treatment, paclitaxel was retained in the uterine cervical cancer tissues for 6 days. To assess the effect of weekly treatment on the retention of paclitaxel, staining for apoptotic cells was carried out on surgical samples (Pt. 1 and Pt. 2). TUNEL-positive cells were detected in uterine cervical cancer tissue after paclitaxel treatment (Fig. 5a, b). In contrast, there were few TUNEL-positive cells in uterine cervical cancer tissue from patients (Pt. 9) who had not undergone paclitaxel treatment (Fig. 5c) and there were no TUNEL-positive cells in uterine endometrium patients (Pt. 1) who had undergone paclitaxel treatment (Fig. 5d). These results indicate that paclitaxel in cancer tissue showed some anti-tumor effects at day 6, whereas in normal tissue it showed none. Indeed, when the paclitaxel concentration was measured in normal endometrium from patients who had been treated with weekly paclitaxel (60 mg/m^2/week) as a NAC, the paclitaxel concentration was below the limit of detection (data not shown).

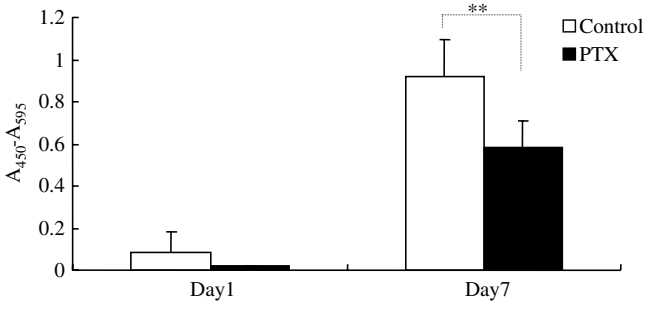

Fig. 3 Effect of paclitaxel on cell growth after 1 and 7 days. CaSki cells were treated with DMSO or 10 ng/ml paclitaxel (PTX) for 16 h, and then the medium was changed and cells were maintained in normal medium. On day 1 or day 7, cells were incubated with BrdU for 24 h, and then the BrdU cell proliferation assay was carried out. The absorbance in each well was measured with a spectrophotometric plate reader at 450 and 595 nm and the results calculated as A_{450}–A_{595}. Data were expressed as the mean ± SD. Differences between groups were tested for statistical significance using Student's *t* test. $P < 0.05$ denotes a statistically significant difference

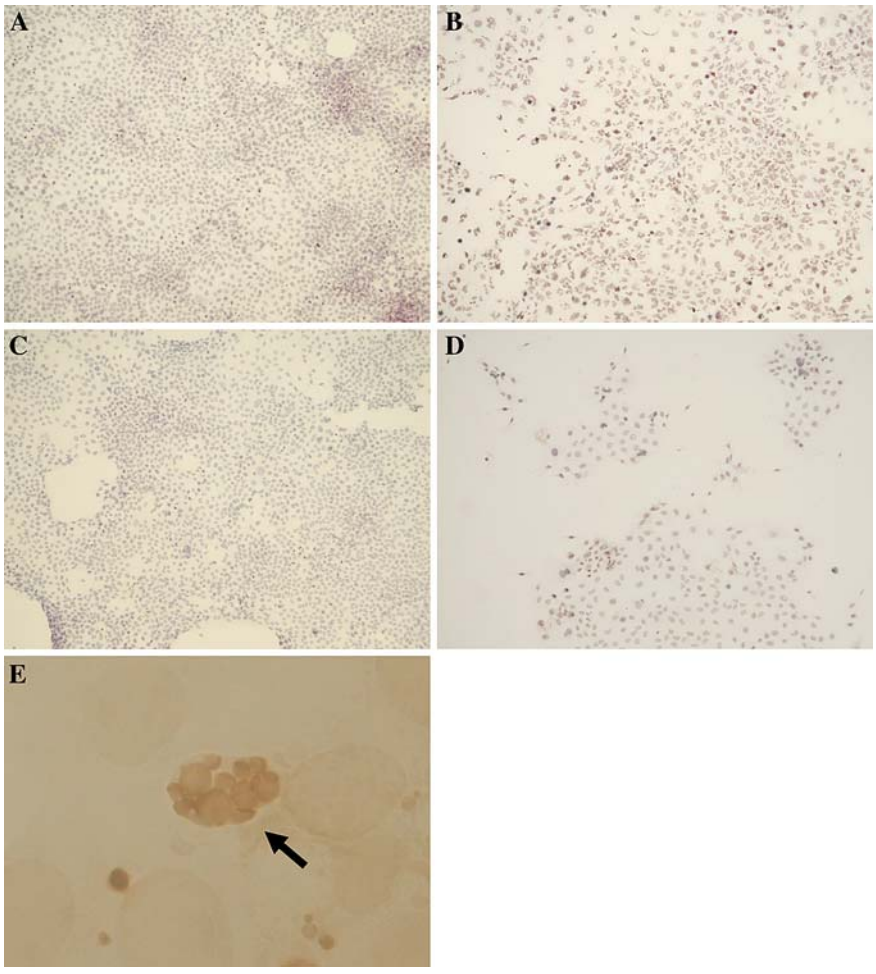

Fig. 4 Effect of paclitaxel on apoptosis after 1 and 7 days. CaSki cells were treated with DMSO (**a**, **c**) or 10 ng/ml paclitaxel (**b**, **d**) and then the medium was replaced with normal medium until day 1 (**a**, **b**) or day 7 (**c**, **d**). Cells were fixed with 4% paraformaldehyde and the TUNEL assay was performed. Nuclei of apoptotic cells stained positive for DAB, while all nuclei stained positive for hematoxylin. (200× magnification). **e** Under high magnification (1,000×), apoptotic cells resulting from paclitaxel treatment showed multinucleation (*arrow*) on day 7

Discussion

Paclitaxel is unusual in that it accumulates in cells and the accumulated drug is retained after washing the cells. Jordan et al. [10] reported that large amounts of accumulated paclitaxel were retained intracellularly following the washing procedure. At 10 nM paclitaxel, the intracellular paclitaxel concentration was 8.3 μM after 20 h of incubation (an 830-fold accumulation), and 48% of the accumulated drug was retained after washing. These workers also reported that in similar experiments performed with vinblastine, most of the vinblastine effluxed from the cells after washing. Our results show that paclitaxel, but not carboplatin, was retained in cells for 24 h following washing. Carboplatin adducts are covalently bound to DNA and are removed by repair mechanisms, which differ from the potentially reversible binding of paclitaxel to tubulin. The paclitaxel sits in a pocket that is lined by several hydrophobic residues and it is situated on the luminal side of the microtuble wall, roughly in the middle of the β monomer along the protofilament direction [24]. Because the aqueous solubility of paclitaxel is low, the paclitaxel concentrations may remain high after washout. Thus, the binding of paclitaxel in cells is not easily reversible and this special feature of retention in cells is characteristic of paclitaxel.

Our results show that the retention of paclitaxel in normal cells was lower than in cancer cells and paclitaxel in normal tissue showed few anti-tumor effects at day 6. Previous studies had demonstrated that paclitaxel was less cytotoxic to contact inhibited normal cells than neoplastic cell lines in assays of growth inhibition and colony formation, suggesting transformed cells were more sensitive [17]. Xu et al. [31] showed paclitaxel with higher specificity for β_{II}-tubulin than for other b-tubulin isotype, irreversibly decreased nuclear β_{II} content, which may be correlated with the cancerous state. It is also reported that normal cells as well as paclitaxel-resistant cell lines may possess α-tubulin or β-tubulin different form that of cancer cells [25], or may overproduce P-glycoprotein compared to cancer cells [23]. Therefore, it is possible that the retention of paclitaxel is low and paclitaxel is less cytotoxic in normal cells.

We found that paclitaxel treatment resulted in tubulin polymerization, and this effect persisted for 24 h after the removal of paclitaxel. Giannakakou et al. [5] showed the same result, that tubulin polymerization induced by paclitaxel was stable for more than 24 h, and treatment of both paclitaxel and vinblastine simultaneously

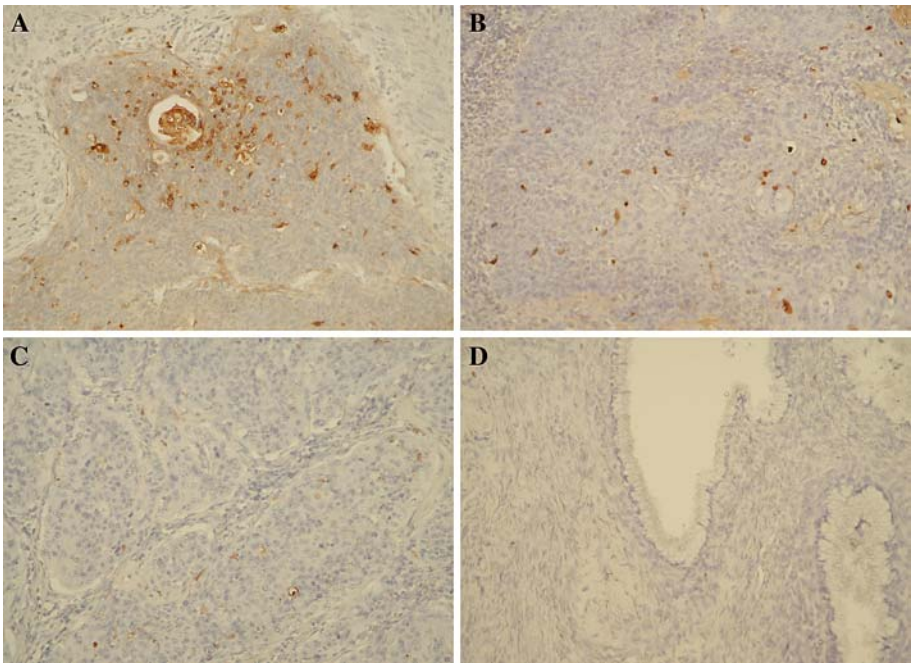

Fig. 5 Apoptosis in uterine cervical cancer tissues at day 6 after cessation of weekly paclitaxel treatment. Patients (Pt. 1 and Pt. 2) were treated with weekly paclitaxel (60 mg/m^2/week) and then underwent radical hysterectomy on the sixth day after the final paclitaxel treatment (**a**, **b**). One patient (Pt. 9) underwent radical hysterectomy without paclitaxel treatment (**c**). A uterine endometrium sample (**d**) was taken from a patient (Pt. 1) who had been treated with paclitaxel. Tissue slices were subjected to TUNEL staining for apoptotic cells. (200× magnification)

diminished paclitaxel-induced tubulin polymerization. We also found that paclitaxel was still retained in cancer cells for 1 week in vivo and in vitro. Taken together, these results indicate that paclitaxel bound to polymerized tubulin and existed in cells for 1 week in vivo and in vitro. An important implication of these results is that the high degree of paclitaxel retention in cells may contribute significantly to the cytotoxicity and antitumor activity of the drug. On the other hand, our result showed a comparison of paclitaxel treatment at day 1 and 7 which reveals that the proportion of cells not affected by the anti-cancer effect of paclitaxel had significantly increased by day 7.

Recently, it has been discovered that the administration of paclitaxel for ovarian cancer on a weekly schedule, rather than the standard every-3-week schedule, might produce greater tumor-cell death [16]. More recently, Green et al. reported that patients with breast cancer receiving a weekly paclitaxel regimen had a higher pathologic complete response rate than patients treated with paclitaxel on an every-3-week program, with improved breast cancer conservation rates [6]. Paclitaxel administered weekly once might be the more active treatment than an every-3-week schedule.

Paclitaxel-induced apoptosis is cell-specific [27], and it depends on the paclitaxel concentration [9] and the duration of the exposure time [12]. The cytotoxicity of paclitaxel is known to operate through at least two main mechanisms. One is the induction of apoptosis as a terminal effect related to a sustained block in mitosis. This pathway is related to JNK (c-Jun N-terminal kinase)/SARK (stress-activated protein kinase) [29], the PKA (protein kinase A)/Bcl2 hyperphosphorylation pathway [26] and Raf-1/Bcl2 phosphorylation [1]. This apoptosis is observed soon after paclitaxel treatment. Continuous exposure to paclitaxel causes mitotic arrest peaking at 24 h [13, 14]. In contrast, the proportion of apoptotic cells steadily increases until the termination of the experiments at 48–60 h [13, 14].

The other mechanism of paclitaxel cytotoxicity takes effect after a transient delay and aberrant exit from mitosis, leading to abnormal chromosome segregation and the induction of apoptosis in a subsequent multinucleated G1-like state. Previous studies [3, 15] report that low concentrations of paclitaxel inhibit the formation of mitotic spindles in cells without arresting the cells in mitosis, and result in multinucleation. Continuous exposure to paclitaxel steadily increases the proportion of apoptotic cells and multinucleated cells until the termination of the experiments at 48–72 h [2, 12, 20]. Woods et al. [30] reported that G1-arrested, multinucleated cells become TUNEL-positive only after several days. Our results suggest that by day 1, many cells had been removed by paclitaxel treatment, consistent with an apoptosis pathway that is fast and triggered by mitotic arrest. On day 7, some cells were still observed as apoptotic cells with multinucleation, consistent with a pathway that is slow and triggered by G1-like arrest. G1-like arrest of the cell cycle is known to depend on the p53 pathway [4, 11, 28]. Paclitaxel-induced multinucleation that was observed in apoptotic cells on day 7 might be p53-dependent [30].

In conclusion, our data show that with weekly paclitaxel treatment, paclitaxel was retained in cancer tissue and cells and, at the same time, apoptosis was observed for 1 week after the cessation of treatment, while the surviving cells grew only slowly. After 2 weeks paclitaxel could not be detected in cancer tissues. The administration of paclitaxel on a weekly schedule, rather than the standard every-3-week schedule, might produce greater tumor-cell death.

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