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Pharmacodynamics of Antitumor Activity of Paclitaxel in Monolayers and Histocultures of Human NSCLC Cells

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ABSTRACT – In this study, we evaluated and compared the pharmacodynamics of paclitaxel (PTX) in human A549 NSCLC cells grown as monolayers or as three-dimensional histocultures. Growth inhibitory effects were determined after incubating cells in drug free medium until 96 hr post drug exposure initiation. Cell cycle arrest and apoptosis were measured by flow cytometry. The growth inhibition induced by PTX was significantly different in monolayers and histocultures, and PTX showed significantly less cytotoxicity in histocultures where large resistant fractions were observed. Moreover, although PTX induced significant G_2/M arrest followed by apoptosis in monolayers in a drug concentration-dependant manner, G_2/M arrest was not elicited in histocultures. However, apoptotic cells appeared from the G_2/M phase in histocultures. In this study, we provide first evidence that PTX in three-dimensional histocultures, does not induce G_2/M arrest, but rather that it induces G_2/M phase specific apoptosis. Overall, our data demonstrate different pharmacodynamics of PTX in traditional monolayer and three-dimensional histocultures.

Key words – Paclitaxel, Histocultures, Cell cycle, Apoptosis

Paclitaxel (PTX) is a chemotherapeutic agent that becomes increasingly important for the treatment of various cancers, especially breast, ovarian, and lung carcinomas.[1] PTX disrupts the normal polymerization/depolymerization cycle of microtubules and thus causes arrest in the G_2/M phase.[2-4] It is generally accepted that the blockade of cell cycle progression in the late G_2/M phase is required for PTX-induced apoptosis.[5-6] However, several lines of evidence produced by recent studies suggest that PTX-induced apoptosis occurs via signaling pathways independent of microtubule stabilization and mitotic arrest.[6]

It is also known that the microenvironment plays a major role in many physiological and pathological events.[7] Due to the lack of an adequate microenvironment, monolayers are an oversimplified model and do not mimic the complex and heterogeneous properties of solid tumors. On the other hand, when tumor cells are grown as aggregates in three-dimensional cultures, they closer recapitulate microenvironmental characteristics *in vivo*. Hence, the use of these two models results in different determined sensitivities to chemotherapeutic agents and has resulted in incorrect predictions of drug efficacy.[8-9] Because of the fundamental discrepancies between these models, three-dimensional culture systems, such as, spheroids and histocultures (cultures of tumor tissue fragments *in vitro*), have received much attention, and are now considered as promising models for therapeutic screening programs. Notably, three-dimensional systems have been recommended as appropriate models for the study of the regulation of invasion and metastasis, angiogenesis, and cell cycle kinetics.[10] In addition, data obtained using histocultures show strong correlations with drug sensitivity and resistance in patients and with clinical outcomes.[11-12]

Although the unique action of PTX against microtubules was discovered in the 1970s,[13] the majority of studies have been performed in monolayer cell culture models, in which mitotic arrest has long been regarded as a hallmark of cellular response to PTX. However, under *in vivo* solid tumor conditions, the antitumor effect of PTX was correlated with apoptotic cell death rather than mitotic arrest,[14] which suggests that the relationship between cell cycle arrest and apoptotic induction under such conditions differs from that in *in vitro* monolayers.

Therefore, the principal aim of this study was to compare the pharmacodynamics of PTX in monolayer and three-dimensional histocultures. Thus, we compared these two culture systems with respect to overall growth inhibition, cell cycle arrest, and apoptosis induced by PTX.

Experimental

Chemicals and Reagents

Paclitaxel (PTX) was provided by the Drug Synthesis &

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Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). Collagen gels (SPONGOSTAN®) were purchased from Johnson & Johnson Medical (Gargrave, UK). Collagenase and other reagents, unless otherwise noted, were purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell culture conditions

The human non-small cell lung cancer (NSCLC) cell line, A549, was purchased from ATCC (Manassas, USA). Cells were maintained in DMEM supplemented with 100 mg/ml streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in 5% (v/v) CO_2 humidified air at 37°C.

Measurement of cytotoxicity in monolayer cultures

Cells were seeded at a density of 1000 cells/well 24 hr prior to drug exposure. They were then treated with various concentrations of PTX (1-2000 nM) for up to 96 hr. Drug-containing medium was removed at the pre-determined times, and cells were further incubated in drug free medium until 96 hr post drug exposure initiation. For example, the response for 6 hr treatment was measured after a 90 hr delay. Cytotoxic effects were evaluated using MTT assays. Briefly, at the end of the incubation period, 20 μl of MTT solution (5 mg/ml) was added. After 4 hr of re-incubation, formazan crystals were dissolved by adding 150 μl of dimethylsulfoxide (DMSO), and absorbance was measured at 540 nm.

Establishment of histocultures

Human NSCLC tumor xenografts were established by injecting 5-6 × 10^6 cells/0.2 ml of serum-free media using a 25-gauge needle subcutaneously into the left and right upper shoulders of Balb/c *nu/nu* mice (Japan SLC, Inc., Shizuoka, Japan). When tumor sizes reached an average size of 800-1000 mm^3, tumor-bearing mice were sacrificed by cervical dislocation and tumors were harvested. Necrotic central portions were removed and non-necrotic portions were cut into 0.5-1 mm^3 fragments using surgical blades. Four or five fragments were placed on a 1 cm^2 piece of pre-wetted collagen gel in 24-well plates. The culture media used were same as used for in monolayers except for the addition of 90 μg/ml of cefotaxime sodium. For histological examinations, tumor pieces were embedded in Tissue Tek OCT compound (Sakura Finetek, Tokyo), using liquid nitrogen, and 5 μm sections were subjected to H&E staining.

Measurement of cytotoxicity in histocultures

Drug treatment was done after 3 days of pre-incubation using the same protocol as described above for monolayers. The MTT assay designed for histocultures by Furukawa *et al.*[15] was adopted with some minor modifications. Briefly, after drug treatments, 100 μl of 0.06% collagenase and 100 μl of 0.2% MTT solution were added to each well and incubated for 20 hr. Media were aspirated carefully, and 1 ml of DMSO was added to each well to dissolve the MTT-formazan. Four hours later, 100 μl of MTT-formazan solution was transferred from each well to a 96-well microplate and absorbances were measured at 560 nm.

Measurement of cell cycle distribution and apoptosis

To determine cell cycle distributions, cells were collected after trypsinization into a single cell suspension, fixed with cold ethanol, and kept at 4°C for 1 hr prior to being stored at -20°C until required for analysis. Upon analysis, fixed cells were washed and resuspended in 1 ml of PBS containing 50 μg/ml RNase A and 50 μg/ml EtBr. After incubating for 20 min at 37°C, cells were analyzed for DNA content by flow cytometry (FACSVantage™, Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10,000 events were acquired and cell cycle distributions were determined using cell cycle analysis software (Modfit®, Verity, Topsham, ME). To simultaneously determine cell cycle contents and apoptosis levels, TUNEL/PI staining was using an Apo-Direct™ kit (Pharmingen, SD). Briefly, after harvesting cells as described above, they were fixed in 1% paraformaldehyde/PBS on ice for 15 min and resuspended in 70% ice-cold ethanol. They were then incubated in 50 μl of reaction buffer containing terminal deoxynucleotidyltransferase and FITC-conjugated dUTP deoxynucleotides (1:1) for 2 hr at 37°C in the dark. After washing, the cells were treated with 5 μg of propidium iodide and 10 Ku of RNase and subjected to flow cytometric analysis using FL1 (FITC) and FL2 (propidium iodide). 10,000 events were analyzed for each sample using CELLQuest software.

Data Analysis

Percent cell growth was defined as % absorbance versus the control and IC_{50}'s were calculated using an E_{max} model (Eq. 1).

$$\%\ growth = (100-R)\times\left(1+\frac{[D]^m}{K_d^m+[D]^m}\right)+R \qquad \text{(Eq. 1)}$$

where D is the drug concentration, Kd is the concentration of drug that results in a 50% reduction in absorbance (i.e., IC_{50}), m is the Hill-type coefficient, and R is the residual unaffected fraction (the resistant fraction).

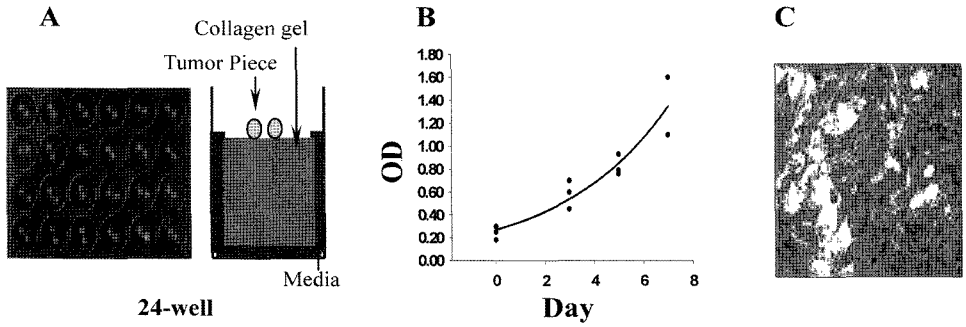

Figure 1–Establishment of histocultures of A549 human tumor xenografts. (A) Schematic diagram of histocultures in 24-well plates. (B) The growth curve of histocultures determined by MTT assay up to 7 days after plating. (C) H&E stained frozen sections of A549 histocultures.

Statistics

Data are expressed as means ± S.D., and the Student's t test was used to determine statistical significance. *P* values of < 0.05 were considered to be statistically significant.

Results

Establishment of histocultures of human tumor xenografts

The schematic representation of a representative histoculture procedure on collagen gel is provided in Figure 1A. Histocultures of A549 human lung tumor xenografts showed continuous growth over 7 days, as confirmed by MTT assay (Figure 1B). The doubling time of histocultures was about 3 days, which was about three times that in monolayers. Histologic examinations revealed that tumor pieces grew well on collagen gels and maintained heterogeneity (i.e., tumor cells and extracellular matrix) and tissue architecture.

Pharmacodynamics of the growth inhibition induced by paclitaxel

Dose-response curves for growth inhibition induced by PTX in A549 cells grown as monolayers and histocultures are shown in Figure 2, and pharmacodynamic parameters are summarized in Table I. Growth inhibitory effects were found to be dependent on drug concentration and exposure duration (Figure 2A). The IC_{50} of PTX reduced with a longer exposure time up to 24 hr, however, no further reduction was observed until 96 hr. These data indicate that the growth inhibitory effect of PTX is enhanced by increasing exposure duration in monolayers. Figure 2B shows the growth inhibition of A549 cells induced by PTX in histocultures. In contrast to monolayers, large resistant fractions were observed at all treatment conditions, i.e., 62.6%, 42.3%, and 46.5% at 24, 48 and 96 hrs, respectively (Table I). A concentration dependent reduction in % growth was observed only between 10 nM and 100 nM, and increased exposure duration to PTX did not induce further growth

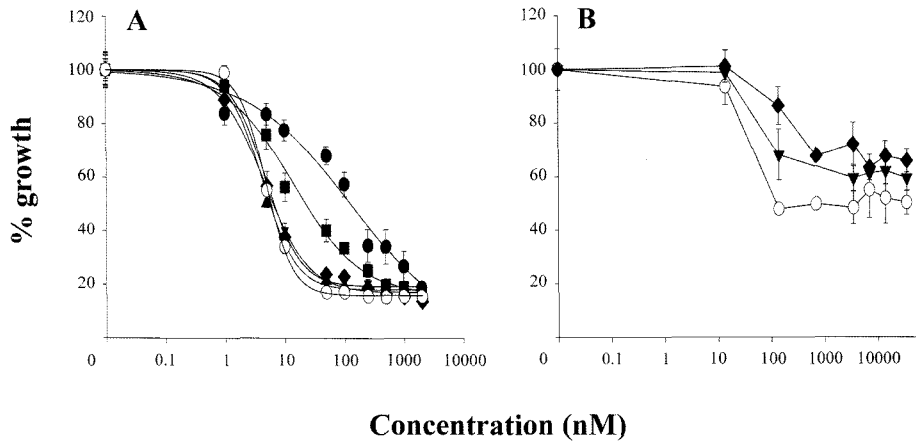

Figure 2–Dose-response curves of PTX in A549 cells grown in monolayers (A) or histocultures (B). MTT assays were done after incubating cells in drug free medium until 96 hr post drug exposure initiation. Symbols are ●, 6 hr; ■, 12 hr; ◆, 24 hr; ▼, 48 hr; ▲, 72 hr; ○, 96 hr.

Table I–*Pharmacodynamic Parameters for the Cytotoxicity of PTX in A549 Cells Grown in Monolayers or Xenograft Histocultures*

	Parameters	Treatment Time (hr)					
		6	12	24	48	72	96
Monolayers	IC_{50} (nM)	228.8±11.6[a]	23.9±2.4[a]	7.8±2.1	8.3±2.0	6.0±2.0	5.3±2.0
	E_{max} (%)	80.4±2.70	82.7±2.71	84.7±1.55	88.5±6.67	89.7±6.7	91.1±6.1
Histocultures	IC_{50} (nM)	ND	ND	ND	ND	ND	ND
	E_{max} (%)	ND	ND	37.4±4.53[b]	39.6±8.7[c]	ND	53.5±6.7

Value represent means±S.D. of three independent experiments.
ND, not determined.
[a], $P<0.001$ compared with IC_{50} values of treatment for ≥ 24 hr.
[b,c], Differs from the corresponding Emax value after 96 hr of exposure in histocultures, $p < 0.01$ and < 0.05, respectively.

inhibition. These data indicate that the growth inhibitory activity of PTX in histocultures is significantly lower than in monolayers. When exposed for 24 hr, 10 nM was enough to induce 60% growth inhibition in monolayers. However, no growth inhibition was observed in histocultures.

Cell cycle arrest and apoptosis induced by paclitaxel

PTX-induced cell cycle arrest and its relationship with apoptosis induction were examined after exposing A549 monolayers and histocultures to PTX for 48 hr and 96 hr, respectively (Figure 3 and 4). Cell cycle distributions with-

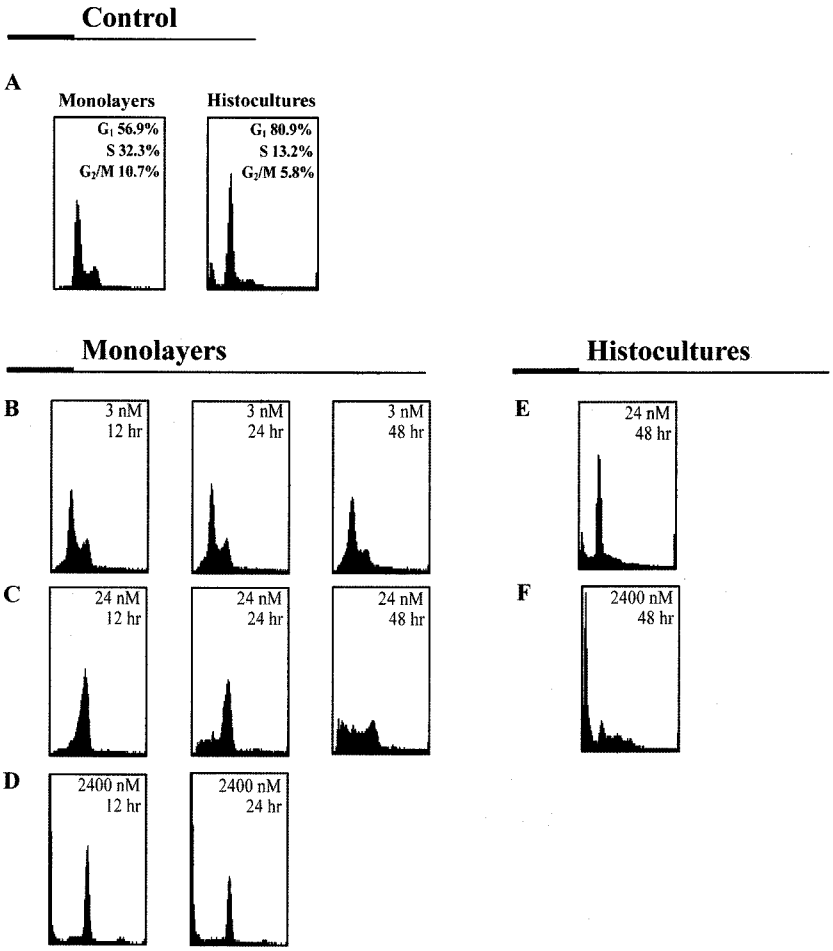

Figure 3–The cell cycle distribution of A549 cells grown in monolayer or histocultures. (A) Representative DNA histograms without drug treatment. Representative DNA histograms showing the cell cycle effects induced by PTX in A549 cells grown in monolayers (B, C, D) and in histocultures (E, F).

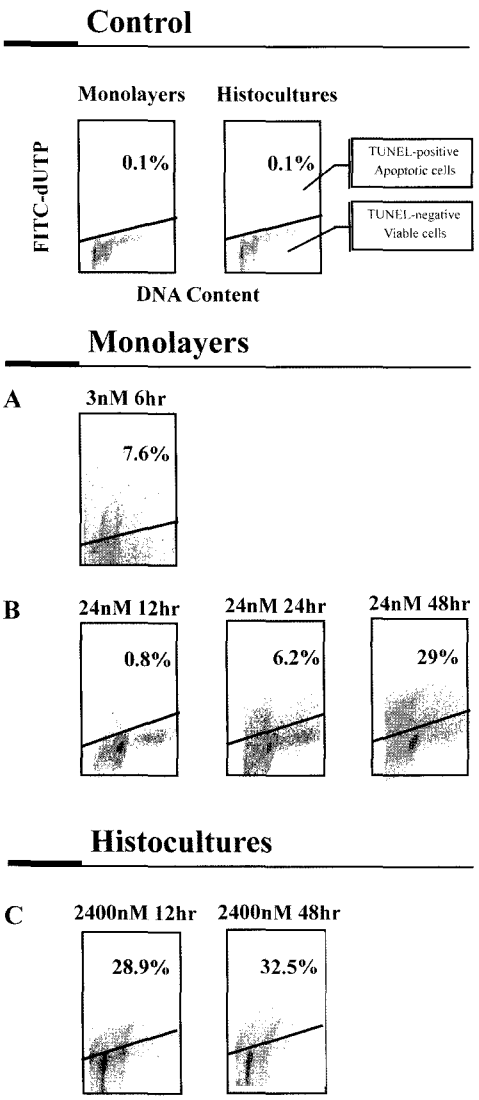

Figure 4–Simultaneous analysis of the cell cycle arrest and apoptosis induced by PTX in A549 cells grown as monolayers (A, B) or histocultures (C). Multi-color density plots constructed after the bivariate analysis of DNA histograms obtained using FITC-dUTP (TUNEL labeling intensity) and PI (DNA content) double-stained samples.

out drug exposure were significantly different for monolayers and histocultures, i.e., in histocultures, the G_1 phase increased by 42.2%, and the S and G_2/M phases decreased by 59.1% and 45.8%, respectively, versus monolayers. In monolayers exposed to a low concentration (3 nM) of PTX, G_1 phase arrest (76.7%) was observed (Figure 3B). In contrast, exposure to a higher concentration of PTX induced G_2/M phase arrest, i.e., 70% and 84% of cells were arrested in the G_2/M phase after 12 hr exposure to PTX at 24 and 2400 nM, respectively (Figures 3C and 3D). In histocultures, G_1 phase abrogation without G_2/M arrest was observed on increasing PTX concentration from 24 nM to 2400 nM (Figures 3E and 3F), which resulted in a significant proportion of hypodiploid cells up to 52% after 96 hr at 2400 nM (data not shown). Bivariate analysis of the cell cycle and apoptosis showed that TUNEL-positive cells appeared from the G_1 population and from the G_2/M phase after treatment with 3 nM of PTX for 6 hr in monolayers (Figure 4A). When exposed to 24 nM, G_2/M arrest and polyploid cells (> 2n) were observed after 12 hr, and cells arrested in the G_2/M phase became TUNEL-positive (apoptotic) after 24 hr (Figure 4B). In histocultures, most apoptotic cells were from the cell population of G_1 and G_2/M phases as was the case for monolayers (Figure 4C).

Discussion

In this study, we evaluated the pharmacodynamics of PTX in human NSCLC cells grown as monolayers or as three-dimensional histocultures. In contrast to the majority of traditional cell culture systems, three-dimensional culture systems are considered to recapitulate many aspects of cell homeostasis and to reflect *in vivo* tumor biology.[8-10] In the present study, we established histocultures of A549 human NSCLC xenografts, and confirmed histocultures growth rates by using MTT-assays (Figure 1). Moreover, we found that the growth characteristics of histocultures differed from those of monolayers, i.e., growth rates (Td: 21 hr vs 72 hr) were significantly lower and G_1 populations (56.9% *vs* 80.9%) were significantly higher (Figures 1B and 3A).

In fact, our study also demonstrates significant differences between the pharmacodynamics of the cytotoxicities induced by PTX in monolayers and histocultures, i.e., the cytotoxicity of PTX in histocultures was significantly lower (Table I). Multicell structure-related chemoresistance to PTX is well documented. A number of studies have shown that PTX is significantly less cytotoxic when tumor cells are exposed to PTX as small multicellular aggregates. Moreover, spheroids (another three-dimensional culture system) of MCF-7 human breast carcinoma cells showed significantly lower sensitivity to PTX than cells in monolayers.[16] In addition, PTX treatment was found to induce the phosphorylation of Bcl-2 in monolayers, but not in spheroids,[17] and HEY and A2780 human ovarian carcinoma cells treated with PTX showed a hypodiploid DNA peak when in monolayers, but not in spheroids.[18] And, the poor penetration of PTX into histocultures has already been reported.[16,19] Therefore, the limited cytotoxicity of PTX in histocultures of A549 xenografts of the present study may be attributed to a penetration barrier as well as

reduced growth rate. Further study is necessary to identify the other intracellular signaling factors of multicell structure-related chemoresistance to PTX.

In general, the cellular target of PTX has been identified as the tubulin/microtubule system. PTX inhibits microtubule depolymerization and promotes the formation of unusually stable microtubules, which results in abnormal microtubule function and a cell cycle block at the late G_2/M phase, which probably results in apoptosis.[2-6,18] Moreover, the induction of mitotic arrest by PTX was found to greatly enhance radiation sensitivity *in vitro*.[20-22] However, several studies have demonstrated that PTX, without mitotic block, is able to induce internucleosomal DNA fragmentation and the typical morphological features of apoptosis in a variety of tumor cell lines, including human leukemia and solid tumor cells,[23,24] indicating that apoptosis and mitotic block can occur independently via a signaling pathway independent of mitotic arrest.[6,25,26] On the other hand, the determinations of relative contributions made by PTX-induced mitotic arrest and apoptosis to its antitumor effect could promote the combinatorial use of PTX and other agents. Alternatively, 5-fluorouracil could significantly repress the cell-killing activity of PTX in human breast and other solid tumor cell lines by preventing tumor cells from entering the G_2/M phase.[27] However, it has not been demonstrated whether antagonism takes place under *in vivo* 3D conditions. For this purpose, we used histocultures to characterize the cell cycle and apoptosis effects of PTX. Our data demonstrated that PTX-induced G_2/M block is dependent on drug concentration and culture condition, e.g., no G_2/M block occurred at 3 nM (Figure 4B) in monolayers and at all concentration ranging from 24 to 2400 nM in histocultures. Our data indicate that in the 3D histoculture system PTX does not induce G_2/M block, but that it nevertheless apoptosis in the G_2/M phase (Figure 3 and 4).

Overall, our data demonstrate that the pharmacodynamics of PTX are markedly different in traditional monolayer cultures and histocultures. The present study also provides first evidence that PTX treatment does not induce G_2/M arrest in 3D histocultures, but rather that it induces apoptosis in the G_2/M phase.

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