SPANDIDOS PUBLICATIONS

Differential regulation of the cytotoxicity activity of paclitaxel by orobol and platelet derived growth factor in human ovarian carcinoma cells

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Abstract. Paclitaxel (PX) binds to and stabilizes tubulin, preventing depolymerization, and resulting in cell death. Based on a previous report showing the activity of phosphatidylinositol kinase (PIK) on tubulin, we investigated the effect of the PI4K inhibitor orobol and the PI3K activator platelet derived growth factor (PDGF) on PX sensitivity. Drug sensitivity was examined by classical colony forming assay. Tubulin isotype expression was determined by semi-quantitative RT-PCR. Microtubule texture was observed by laser confocal microscope using anti-ß-tubulin antibody. Apoptotic activity was estimated by frequency of condensed nuclear chromatin with Hoechst 33342 stain. Orobol enhanced PX sensitivity of human ovarian carcinoma 2008 cells by 18.9±1.2-fold (N=3; P<0.01). In contrast, pretreatment with PDGF rendered cells resistant to PX by 2.3±0.4-fold (N=3; P<0.01). Neither orobol nor PDGF showed any effect on cell growth. Orobol produced a 2.5-fold sensitization in cisplatin-resistant 2008/C13*5.25 (C13) cells, and PDGF rendered the cells 2.3-fold resistant to PX. Orobol suppressed the ß4a-tubulin isotype expression by 85% and other isotypes by 20%. In contrast, PDGF induced ß4a-tubulin isotype expression by 1.3-fold, while it supressed all the other isotypes by 20-40%. Orobol produced thick microtubules and PDGF generated ring condensed microtubules. Orobol promoted PX-induced apoptosis, while PDGF caused 50% reduction of apoptosis. These results indicate that orobol and PDGF regulate PX sensitivity by reciprocally altering the proportion of tubulin isotype expression and PX-induced apoptotic signaling.

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Abbreviations: IC_{50}, drug concentration causing 50% inhibition of colony formation; PIK, phosphatidylinositol kinase; PDGF, platelet derived growth factor

Key words: paclitaxel, tubulin, drug sensitivity, orobol, PDGF

Introduction

The antimicrotubule agent paclitaxel (PX) has shown some efficacy in the treatment of ovarian and metastatic breast cancers, and particularly encouraging is its utility in advanced ovarian cancers that are refractory to DNA damage-based chemotherapy (1). In contrast to tubulin depolymerizing toxins such as nocodazole or colcemid, PX stabilizes microtubule formation and, in continuous treatment, it prevents completion of mitosis, resulting in cell-cycle blockage in mitosis and activation of apoptosis. PX reversibly binds to microtubules *in vitro* and apparently does not bind to free tubulin (2,3). PX strongly stimulates the rate and extent of microtubule polymerization and reduces the concentration of soluble tubulin (4). Additionally, PX also inhibits tubulin exchange at microtubule ends and reduces the fluxral rigidity of them (5). At subnanomolar concentrations, PX suppresses microtubule dynamics by affecting shortening of microtubules (6). At higher concentrations, PX suppresses dynamics by inhibiting the growing and shortening of microtubules (7). Little is known about how microtubule dynamics are regulated in cells. *In vitro* and in cells, microtubule ends switch between states of growing and shortening, a process known as 'dynamic instability', apparently due to the gain and loss of a stabilizing GTP- or GDP-Pi-liganded tubulin cap at the microtubule ends. Net growing of microtubules can occur at one microtubule end and net shortening can occur at the opposite end, a process termed 'treadmilling' or 'flux'. A possible mechanism for control of microtubule polymeriztion dynamics could involve the isotypic composition of the tubulin itself. Recent evidence indicates that some tubulin isotypes may have distinct functions. Falconer *et al* (8) found that the αßII isotype was incorporated into a colchicine-stable subset of microtubules, whereas the αßIII isotype was preferentially incorporated into a colchicine-labile microtubule subset in neuronal cells.

Resistance to PX has been shown to be mediated by the drug efflux pump, P-glycoprotein, but could also result from alterations in its intracellular target, tubulin. Although precise molecular alterations in microtubules have not been identified, several mechanisms have been proposed. These include decreased tubulin content (9), point mutations as evidenced by altered migration of α- or ß-tubulin, differential expression of ß-tubulin isotypes (10,11). Haber *et al* (10) and other investigators (11,12) have reported altered expression of the

ß-tubulin isotype in taxol-resistant cells. The differential expression and high conversion of isotypes have raised the question of whether isotypes provide unique biochemical properties that result in functional differences in microtubules. *In vitro* studies using purified brain tubulin have indicated that there are functional differences among the isotype classes in microtubule dynamics, assembly and drug binding.

Phosphoinositide turnover plays an important role in a cellular response to a wide variety of metabolic stimuli. Phosphatidylinositol (PI) 4-kinase (K) is a broadly distributed enzyme, and has been associated with the response to growth factors (13), oncoproteins (13) with tyrosine kinase activities. PI4K activity is associated with neoplastic proliferation (13) and was shown to be able to regulate cisplatin sensitivity (14). On the other hand, Kapeller *et al* have found evidence suggesting an interaction between PI3K and microtubules (15) and they have also reported that PI3K binds constitutively to α/ß-tubulin and binds to γ-tubulin in response to insulin (16). Moreover, the signal transduction cascade initiated by the activation of PI3K is implicated in an anti-apoptotic signaling generated by growth factors. Collectively, these data suggested that the drugs having an effect on PIK might have the potential to alter tubulin composition and to regulate PX sensitivity.

Materials and methods

PX was obtained from Bristol-Myers Squibb K.K., Japan. Orobol was kindly donated by Dr Umezawa at Keio University of Yokohama, Japan. PDGF was purchased from Sigma Chemical Co., St. Louis, MO.

Tumor cell lines. The human cell line 2008 was established from a patient with a serous cystadenocarcinoma of the ovary (17). A resistant subline, designated 2008/C13*5.25 (C13), was obtained by 13 monthly selections with 1 μM cisplatin followed by chronic exposure to cisplatin increased stepwise to 5 μM (18). The cells were cultured at 37˚C in 95% air and 5% CO_2. They were grown in RPMI-1640 medium supplemented with 5% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Orobol or PDGF treatment and colony assays. Colony forming assays were used to assess the effect of orobol/PDGF on PX sensitivity. After 90-min preincubation in the presence or absence of 0.1 mM of orobol, cells were treated with PX for 2 or 24 h. For the assessment of PDGF effect, cells were preincubated in 0.5% serum medium for 12 h and serum-starved cells were incubated in the presence or absence of 40 ng/ml PDGF for 2 h. To determine the optimal condition for orobol/PDGF treatment, cells were treated with either of them for different incubation times and concentrations. Cells were then treated with PX for 2 or 24 h. The drug-containing medium was aspirated and replaced with drug-free medium. After 10 days plates were fixed and stained. Colonies of over 60 cells were counted macroscopically. The IC_{50} was defined as the drug concentration reducing the number of colonies by 50% and was determined by fitting the dose-response curve to Hill model equation (19) using computer soft program Igor Pro 4.0 (WaveMetrics, Inc., OR, USA).

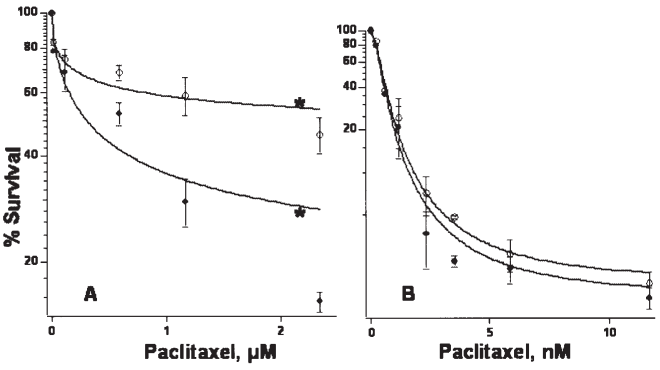

Figure 1. Effect of orobol on PX sensitivity of 2008 cells. Cells were pre-incubated for 90 min in the presence (•) or absence (○) of 0.1 mM orobol followed by either 2-h (A) or 24-h (B) exposure to PX. Points, mean values of 3 experiments performed with triplicate cultures; bars, SD. Orobol increased PX sensitivity in 2-h exposure but it did not alter the sensitivity in 24-h exposure.

Detection of apoptosis. Morphological detection and quantification of apoptosis by Hoechst-33342 staining was determined by the method of Waterhouse *et al* (20). The percent induction of apoptosis was estimated by counting the frequency of condensed nuclear chromatin in duplicate samples of 400 cells.

RNA isolation and ß-tubulin isotype transcript analysis by RT-PCR. Total-RNA was isolated from 2008 cells by standard procedure (21). Four micrograms of total-RNA were subjected to reverse transcription with oligo(dT), deoxynucleotide triphosphates, and 200 U superscript II RNase H-reverse transcriptase in a 20-μl reaction mixture. ß-tubulin isotypes were amplified using specific primer pairs as shown below. Actin RNA amplification was performed to check for DNA contamination and was used to normalize RNA roading differences. Thirty-five cycles of PCR were performed and the PCR products were run on a 2% agarose gel and were visualized by ethidium bromide.

The specific primer pairs for each of the ß-tubulin isotype sequences were obtained according to Ranganathan *et al* (11) for ß1-, ß2-, ß3- and ß4b-tubulin or to Kavallaris *et al* (12) for ß4a-tubulin. The sense primer sequence was the same for ß1-, ß2-, ß3- and ß4b-tubulin and was 5'-CAACAGCACGG CCATCCAGG-3'. The antisense primer sequences were: ß1, 5'-AAGGGGCAGTTGAGTAAGACGG-3'; ß2, 5'-GTAGA AAGACCATGCTTGGG-3'; ß3, 5'-CTTGGGGCCCTGGGC CTCCGA-3'; ß4b, 5'-CTTTCCCCAG TGACTGAAGG-3' (11). The sense primer sequence for ß4a-tubulin was 5'-TCT CCGCCGCATCTTCCACC-3', and antisense primer sequence was 5'-CCGGCCTGGATGTGCACGAT-3' (12). The sense primer for ß-actin was 5'-TACATGGCTGGGGTGTTGAA-3' and the antisense primer was 5'-AAGAGAGGCATCCTCAC CCT-3' (22). Fluorescence was imaged with an Olympus IX70 inverted microscope. Images were digitalized and analyzed using NIH Image software (http://rsb.info.nih.gov/nih-image/index.html).

Immunofluorescence and confocal scanning. 2008 cells were plated onto glass coverslips and grown to subconfluency. Seventy-two hours after treatment with either orobol, PDGF

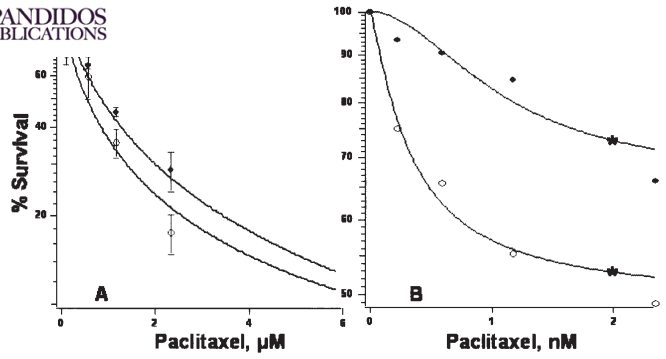

Figure 2. Effect of PDGF on PX sensitivity of 2008 cells. Cells were pre-incubated in 0.5% serum medium for 12 h and serum-starved cells were incubated in the presence (•) or absence (○) of 40 ng/ ml PDGF for 2 h. Cells were then treated with PX for 2 (A) or 24 h (B). Points, mean values of 3 experiments performed with triplicate cultures; bars, SD. PDGF decreased PX sensitivity in 24-h exposure but it did not alter the sensitivity in 2-h exposure.

or PX, cells were rinsed in PBS and fixed in ice-cold methanol. The cells were incubated in 10% calf serum in PBS for 30 min at 37˚C to block non-specific binding of antibodies. Cells were then incubated with primary general ß-tubulin antibody purchased from Sigma Chemical Co. for 1 h at 37˚C at a 1:100 dilution. PBS-rinsed cells were incubated with FITC conjugated anti-mouse IgG (Sigma Chemical Co.) at a 1:100 dilution for 1 h at 37˚C. Cells were rinsed and mounted in 90% glycerol-10% PBS containing 0.2% p-phenylenediamine. Stained cells were scanned with the use of Carl Zeiss LSM410 laser confocal microscopy. Detector sensitivity, black level, detection aperture, and laser power were kept constant for all of the scans.

Statistical analysis. Differences between samples or groups of samples were determined by Student's t-test using two-sided P-values.

Results

Effect of orobol on PX sensitivity. Fig. 1A shows that orobol increased PX sensitivity. After 90-min preincubation in the presence or absence of 0.1 mM of orobol, proliferating 2008 cells were treated with PX for 2 h. In control, cells were treated with vehicle alone for 90 min and then they were exposed to PX for 2 h. The IC_{50}s determined by each dose-response curve were 5.8±0.2 μM (SD) in control, and 0.3±0.1 μM in the presence of orobol. Orobol at concentrations even up to 1.0 mM did not cause any toxicity *per se*. Thus, the interaction between orobol and PX appeared to be truly synergistic. Orobol produced an 18.9±1.2 (SD)-fold (N=4; P<0.005) increase in sensitivity to PX. However, as shown in Table I and Fig. 1B, when 2008 cells were exposed to PX for 24 h, no change in PX sensitivity was observed.

Effect of PDGF on PX sensitivity. The PX dose-response curves for 2008 cells were determined with or without PDGF treatment. Fig. 2B shows that when the 2008 cells were treated with 40 ng/ml PDGF for 90 min, there was a marked decrease in sensitivity to PX in a 24-h exposure schedule. The IC_{50}s of each of these curves were 0.35±0.01 μM (SD) in control and 2.78±0.01 μM (SD) after 2-h PDGF treatment. Thus PDGF caused the cells to become 7.90±0.03 (SD)-fold (N=4; P<0.01) more resistant to PX. A small but not significant change in sensitivity in a 2-h PX exposure schedule was produced by treatment with PDGF (Fig. 2A). These data indicate that PDGF rendered the cells resistant to PX in a schedule-dependent manner.

Regulation of PX sensitivity in cisplatin-resistant C13 cells. The C13 cell line is 10-fold resistant to cisplatin when determined by clonogenic assay. Table I shows that orobol increased cellular sensitivity to PX in C13 variants by 2.5±0.2 (SD)-fold (N=4; P<0.01) in 2-h drug exposure, whereas PDGF rendered the cells resistant by 2.3±0.2 (SD)-fold (N=4; P<0.01) in 24-h drug exposure. Despite the rather small effect, both orobol and PDGF altered the drug sensitivity of cisplatin-resistant variants in the same manner as in 2008 cells.

Induction of apoptosis. PX stimulates the DNA fragmentation characteristic of apoptosis (23). Therefore, we expect PX-induced apoptotic signaling might be involved in the effect of orobol or PDGF. We examined the effect of orobol or PDGF on PX-induced apoptosis by Hoechst-33342 stained nuclear morphology (Fig. 3a). As shown in Fig. 3b-A, orobol produced PX-induced apoptosis by 69%, whereas PDGF treatment after 12-h incubation under 0.5% serum conditions decreased the number of PX-induced apoptotic cells to ~60% compared

Table I. Effect of orobol/PDGF on paclitaxel sensitivity in platinum-sensitive 2008 cells and -resistant C13 cells.

Cell	Drugs	Taxol exposure (h)	Sensitivity		P-value
			Resistant/sensitive	-Fold	
2008	Orobol	2	Sensitive	18.9±1.2	0.034
		24	-	1.0±0.3	-
	PDGF	2	-	1.5±0.3	-
		24	Resistant	7.9±0.3	0.002
C13	Orobol	2	Sensitive	2.5±0.2	0.014
	PDGF	24	Resistant	2.3±0.2	0.001

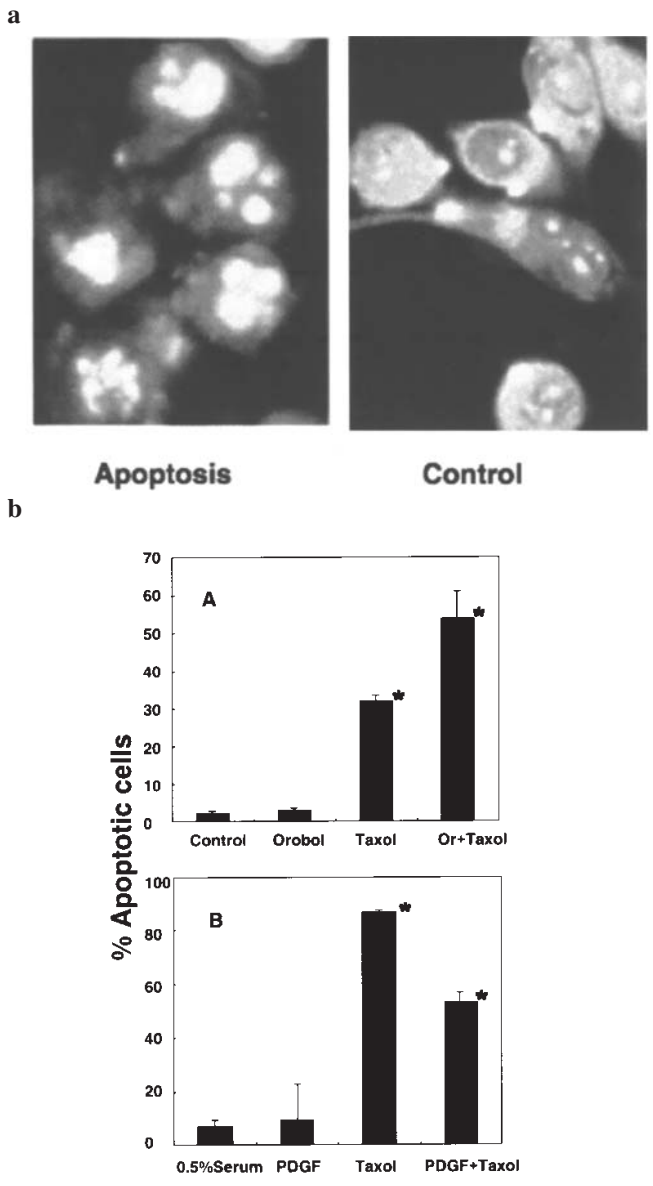

Figure 3. (a) Nuclear condensation and fragmentation was observed in apoptotic cells stained with Hoechst-33342. (b) Effect of orobol and PDGF on apoptotic potential induced by PX. While orobol increased PX-induced apoptosis (A), PDGF significantly inhibited the apoptosis (B). Columns, mean values of 3 experiments performed with triplicate experiments; bars, SD. *P<0.01.

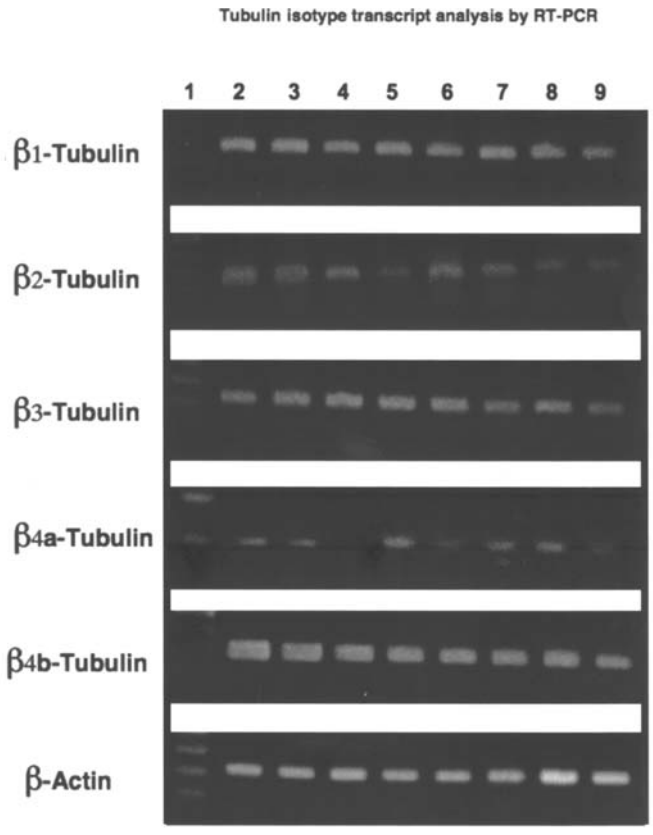

Figure 4. Expression of ß-tubulin isotypes in 2008 cells. RT-PCR was performed 24 or 72 h after incubation with orobol or PDGF. Lanes 2 and 3, control 2008 cells 24 h (lane 2) or 72 h (lane 3) after incubation in medium alone. Lanes 4 and 5, 24 h (lane 4) or 72 h (lane 5) after incubation in medium containing orobol. Lanes 6 and 7, 24 h (lane 6) or 72 h (lane 7) after incubation in 0.5% serum medium. Lanes 8 and 9, 24 h (lane 8) or 72 h (lane 9) after incubation in 0.5% serum medium followed by PDGF containing medium. Photos are representative gels of the three independent PCR reactions.

to PX alone control. Under the condition tested, neither PDGF alone nor 0.5% serum treatment showed any effect on the apoptotic potential. Thus, we concluded that orobol's sensitization effect is at least partly associated with apoptotic signaling pathway while PDGF rendered the cells resistant to PX by inhibiting PX-induced apoptosis.

Analysis of ß-tubulin isotype expression. Kavallaris *et al* (12) previously showed the altered expression of specific ß-tubulin genes in taxol-resistant ovarian tumors. On the basis of those data, we expected that possible overexpression of certain isotypes of the ß-tubulin genes in orobol- or PDGF-treated cells would mimic the effect of them on TX sensitivity. ß-tubulin gene expression was examined in 2008 cells after preincubation in medium containing either orobol or PDGF. The high specificity of isotype-specific primer pairs was confirmed by Kavallaris *et al* (12). PCR involving the control gene, ß-actin, was used for gene expression analysis. This is quite useful for the semi-quantitative analysis of genes in each cell.

Expression of all ß-tubulin isotypes was found in various control 2008 cells (Fig. 4, lanes 2, 3, 6, 7) and those treated with either orobol (Fig. 4, lanes 4 and 5) or PDGF (Fig. 4, lanes 8 and 9) under their optimal cultural conditions. Densitometric analysis of expression levels of each of the isotypes was quantitated relative to the expression of the control gene, ß-actin, by determining the ratio between the target and control gene PCR product (Fig. 5). Fig. 5A showed that ß1, ß2, ß3, and ß4b, all expressed at low levels 24 h after orobol treatment, returned to some extent after 72 h. It should be noted that orobol strongly suppressed ß4a expression by 85% 24 h after treatment but the expression was recovered redundantly after 72 h while ß2 and ß4b expression remained suppressed by 20 or 30%. Likewise, PDGF also suppressed ß1, ß2, ß3 and ß4b by 20-40% 24 h after treatment but all recovered spontaneously after 72 h (Fig. 5B). However, PDGF increased ß4a expression by 28% 24 h after treatment but the expression returned to a normal level after 72 h and all the other subtypes' expression remained suppressed by 10-20%. Consequently, orobol and PDGF appear to reciprocally regulate the expression

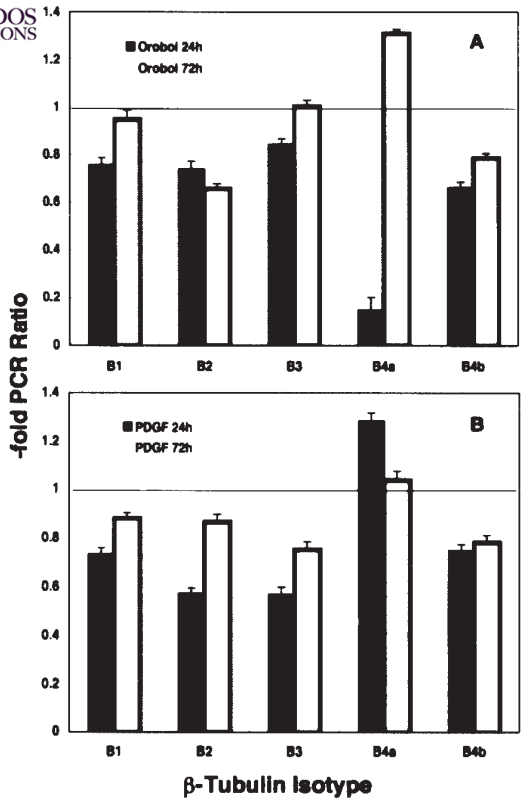

Figure 5. Ratios between the target and control ß-actin gene products were determined by densitometry for each sample. Columns, mean values of 3 experiments; bars, SD.

of ß4a isotype and differentially regulate ß3 expression. The data are mostly consistent with those by Kavallaris *et al* (12) and Ranganathan *et al* (11) regarding PX sensitivity.

Immunofluorescence and confocal scanning. Four types of microtubule structure were observed in differently treated 2008 cells examined by fluorescent microscopy. Orobol-treated cells contained subtly thicker microtubules with more complex structure (Fig. 6B). PDGF-treated cells contained ring condensed microtubules (Fig. 6C). In contrast, cells treated with the stabilizing agents, taxol, have a condensed ball shaped microtubule center (Fig. 6D). These structural differences might be caused by a breakdown of combination balance among ß-tubulin isotype expression patterns induced by orobol or PDGF.

Discussion

This study was conducted on the basis of a report demonstrating the relation between PIK activity and tubulin (16). Our initial hypothesis was that two independent PIK inhibitors, orobol and PDGF, might have the potential to alter the tubulin assembly in association with PX sensitivity and, thus, we have focused more on tubulin composition change rather than PIK signaling pathway.

Unlike other antimicrotubule drugs which induce the disassembly of microtubules, PX promotes the polymerization of tubulin (2,4). PX stabilizes tubulin polymerization resulting in mitotic arrest and apoptotic cell death. PX binds to the N-terminal 31 amino acids of the ß-tubulin subunit in the microtubule, rather than to tubulin dimers (2,24). Altered expression of distinct ß-tubulin isotypes could modify tubulin or/and microtubule dynamics or stability of microtubules in such a way that the action of taxol is diminished. Selection of less stable isotypes could offer cells a survival advantage when exposed to a stabilizing drug such as taxol. PX, at low concentrations (<100 nM), suppresses microtubule dynamics by affecting shortening of microtubules (6). While at higher

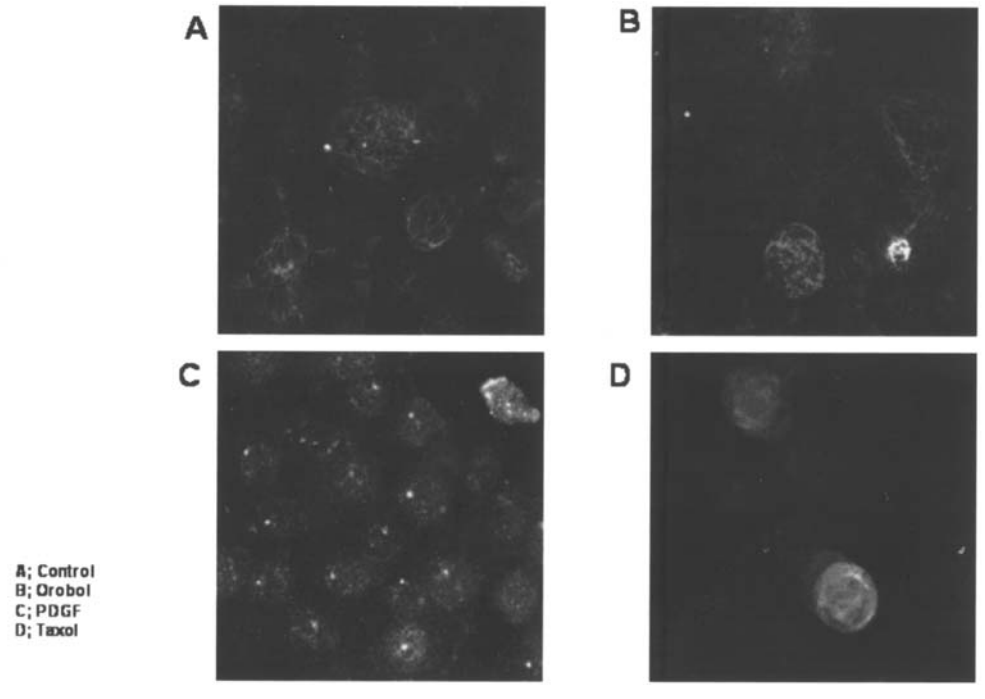

Figure 6. Four types of microtubule structure. (A) Control. (B) Orobol-treated cells contained thicker microtubules with more complex structure. (C) PDGF-treated cells contained ring condensed microtubules. (D) Taxol-treated cells have a condensed ball shaped microtubule center.

concentrations between 100 nM and 1 μM, PX suppresses dynamics by inhibiting the growing and shortening of microtubules. Our data demonstrated that orobol produced PX sensitivity in 2008 cells and its cisplatin-resistant derivatives when they are exposed to higher concentrations of PX but not to lower concentrations of PX. Orobol suppressed all isotypes of ß-tubulin with the strongest effect on ß4a and caused the structural changes in microtubles resulting in increased levels of apoptosis although the rate of apoptotic cells did not correspond well to the level of the orobol-sensitization effect. While ß3 tubulin overexpression is highlighted in PX-resistant cells in some studies (25-27), the lower level of ß1, ß3 and ß4a in PX-sensitive cells is consistent with the data of Kavallaris *et al* (12) and Shalli *et al* (28). They documented that PX-resistant cells displayed a significant increase in ß1, ß3, and ß4a isotypes. Although the data shown here are insufficient to demonstrate the true mechanism of schedule dependency of the orobol-sensitization effect, we offer the following speculation. First, because the action mechanism of PX is concentration-dependent, the orobol-induced altered expression pattern of ß-tubulin isotypes with modification of microtubule structure might be associated with the mechanism specific for PX. According to Derry *et al* (6), PX binding to microtubules approaches saturation at higher concentrations of PX and microtubule dynamics are nearly completely shut down and microtubule polymer mass increases sharply. Under those conditions, the alteration in polymerization pattern or dynamics of α/ß-tubulin dimer caused by alteration of isotype expression may not be further modulated. The only possible mechanism is the changing in tubulin crystal structure which might facilitate the binding affinity of PX even to the internal core region of microtubules as is explained by Derry *et al* (6). The evidence that PX does not bind to solubule tubulin also supports this hypothesis.

In contrast, PDGF decreased PX sensitivity in those cells when they are exposed to lower concentrations of PX but not when exposed to higher PX concentrations. The effect was not specific to the cell line that we used but was also noted in other cells such as in NIH3T3 cells (data not shown). PDGF increased ß4a isotype expression significantly, which is the opposite effect to orobol, however it decreased the expression level of all the other isotypes probably resulting in microtubule structural change. PDGF also significantly decreased the apoptotic cell number. At the lowest effective PX concentrations, the binding of PX to microtubules potently suppresses the rate of shortening with some effects on the microtubule polymer mass (6). However, at this concentration level, PX also inhibit mitosis without increasing the microtubule polymer mass (29). Although the precise mechanism of PDGF's effect on PX sensitivity could not be clarified from our available data, evidence shown by others strongly suggested that the effect of PDGF is not necessarily associated with the tubulin-isotype specific polymerization properties which might be altered by different isotype expression patterns caused by PDGF. The so-called PI3K/Akt pathway is activated in response to a mediator of growth factor-related survival signals such as PDGF (30). Activation of this PI3K/Akt survival pathway protects against apoptotic stress stimuli including PX exposure. One might expect the effect of PDGF to be associated with activation of this signaling pathway.

We have shown here the possibility of controlling PX sensitivity. One of the advantages of this principle is that both orobol and PDGF are non-toxic to humans in clinical settings. PDGF is derived from platelet and was detected in serum samples. Orobol is one of the derivatives of isoflavone and should be non-toxic. The challenge is to make the locoregional tumor cells more sensitive to PX, while rendering the normal bone marrow cells more resistant to protect themselves from PX toxicity.

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