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Involvement of protein kinase C in taxol-induced polyploidization in a cultured sarcoma cell line

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Abstract

Taxol was found to inhibit the proliferation and to induce the polyploidization of cultured methylcholanthrene-induced sarcoma cells (Meth-A cells). To investigate whether protein kinase C is involved in taxol-induced polyploidization, phorbol 12-myristate 13-acetate (PMA), which regulates the activity of protein kinase C, was used along with taxol to treat the cells. We found that PMA did not interfere with the proliferation and did not induce polyploidization by itself. However, at low concentration, taxol, which by itself did not induce polyploidization, clearly induced polyploidization in the presence of PMA. To explore the mechanism by which PMA potentiates polyploidization, the levels of the G1 checkpoint-related proteins cyclin E and cdk2, and those of the G2 checkpoint-related proteins cyclin B and cdc2 were determined by flow cytometry. We found that both G1 and G2 checkpoint-related proteins increased during the induction of polyploidization. To verify the relationship between protein kinase C and tubulin polymerization, flow cytometry was used to determine the total content of tubulin protein, and morphological observation was used to examine spindle organization. PMA did not affect the taxol-induced increase in tubulin protein, but markedly potentiated taxol-induced spindle disorganization. These findings suggest that protein kinase C plays an important role in regulating the induction of polyploidization in Meth-A cells.

Keywords: Taxol; Polyploidization; Phorbol 12-myristate 13-acetate (PMA); Protein kinase C; Polymerization

1. Introduction

Microtubules are critical components of the cytoskeleton in eukaryocytic cells and are involved in many cellular processes, including such diverse functions as morphogenetic events to maintain shape and polarity (White and Rao, 1998), chromosome migration during mitosis (Nedelec et al., 1997; Straight et al., 1997), intracellular organelle transport (Sheetz, 1996), and cell motility (Canman and Bement, 1997). Microtubular structures, such as spindle and cytoplasmic microtubules, are formed by the assembly of the ∂-β-tubulin heterodimer. Microtubular dynamics are regulated by tubulin polymerization and depolymerization, which provide the structural basis for the mitotic cycle (Severin et al., 1997).

Taxol (paclitaxel), identified by Wani et al. (1971), is a diterpenoid plant product isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) and is one of the most effective antitumor agents (Fig. 1). Taxol has shown promising effects against various types of cancer, such as refractory malignant tumors originating or forming metastatically in lung, ovary, breast, head, and neck (Cowden and Paterson, 1997). It is known that the efficacy of taxol is dependent on its specific inhibition of the normal function of cellular microtubules and mitotic spindles, resulting in blockage of the cell cycle at the metaphase/anaphase transition and cell death (Ling et al., 1998; Torres and Horwitz, 1998; Wang et al., 1998b). In contrast to other antimicrotubule agents such as nocodazole, colcemid, vincristine, and vinblastin, which antagonize tubulin monomer polymerization and induce the depolymerization of tubulin polymers into monomers, taxol

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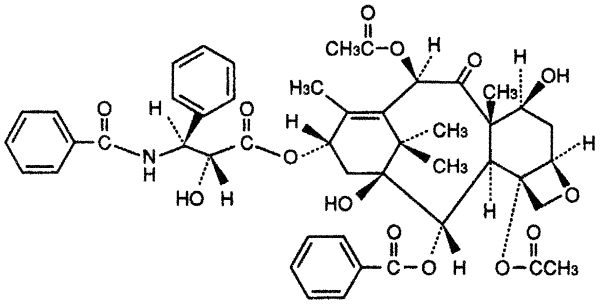

Fig. 1. Molecular structure of taxol.

increases the rate of tubulin monomer polymerization and stabilizes the microtubule cytoskeleton against depolymerization after it is formed (Arnal and Wade, 1995).

Protein phosphorylation is catalyzed by a multitude of protein kinases and is one of the most important mechanisms in regulating diverse cellular functions by transmission of a wide variety of extracellular and intracellular signals. Among the signal transduction pathway mediators, protein kinase C is one of the most widely studied kinases and has been shown to be a starting point for a kind of signal transduction involving protein kinases which affect the cell cycle (Frey et al., 1997; Livneh and Fishman, 1997). Furthermore, protein kinase C participation in cytoskeletal organization, including microtubule dynamics, has also been reported (Lehrich and Forrest, 1994; Garcia-Rocha et al., 1997).

Polyploidization is a complex process involving several distinct steps: escaping from the mitotic block, bypassing cellular division, entering into a new cycle of DNA replication, and characteristically inducing the formation of polyploid cells with a DNA content $>4C$ (C = haploid DNA content) (Zong et al., 1994). The cell division cycle during eukaryotic cell proliferation proceeds by a defined sequence of events where progression to the next phase depends upon completion of the preceding one. Checkpoints, including the G1 checkpoint, G2 checkpoint, and mitotic checkpoint, controlling this progression have been identified (Muhua et al., 1998; Nurse et al., 1998; Orr-Weaver and Weinberg, 1998; Pines, 1999). Although the exact mechanism by which polyploidization is induced remains obscure, it is evident that checkpoint dysfunction is responsible for the induction of polyploidization (Zong et al., 1995).

Although clinical success using taxol in cancer chemotherapy has been achieved, the development of drug resistance (Duan et al., 1999; Kavallaris et al., 1999) and side effects (Cavaletti et al., 1995) impede the effective use of taxol. In an effort to explore possible drug combinations with taxol that would allow the use of lower concentrations of taxol while maintaining efficacy, phorbol 12-myristate 13-acetate (PMA) was used to investigate the relationship between protein kinase C and the effect of taxol. We found that PMA acts synergystically with taxol to induce polyploidization by increasing the expression of G1 and G2 checkpoint-related proteins and by potentiating taxol-induced spindle disorganization. These findings suggest that regulation of protein kinase C activity may mediate the effects of the combination in vivo.

2. Materials and methods

2.1. Materials

Taxol and PMA were purchased from Sigma and dissolved in dimethyl sulfoxide. Taxol was stored in the dark at 4°C and PMA was stored at −20°C. The antibodies against mouse cyclin B, cdc2, cyclin E, cdk2, and tubulin used in this study were purchased from Wako Pure Chemical, Tokyo. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Cell line and culture conditions

Methylcholanthrene-induced sarcoma cells (Meth-A line, syngeneic to BALB/c mice) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 50 u/ml of penicillin and 50 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The viability of the cells used in these experiments was consistently more than 95% when evaluated by the Trypan blue exclusion method.

2.3. Measurement of cellular parameters

The number and volume distribution of Meth-A cells were determined using a Coulter counter model ZM and a Channelyzer model 256 (Coulter Electronics). The analyzer was calibrated using 9.61-μm styrene beads.

2.4. Cell cycle analyses

Flow cytometry was used to determine the DNA content of Meth-A cells. The cells were fixed in 70% ethanol, and then treated exhaustively with pancreatic RNase A and stained R with propidium iodide (10 μg/ml in phosphate-buffered saline). Fluorescence from individual cells was measured with a flow cytometer (Cytofluorograf system 50H, Ortho Instruments) and a FACSort (Becton Dickinson Immunocytometry System). The relative intensities of red fluorescence were measured and DNA histograms were obtained. The results were analyzed using CELLQuest software.

2.5. Assay of relative protein content by flow cytometry

Meth-A cells were collected, washed in phosphate-buffered saline, fixed with 1% paraformaldehyde for 30

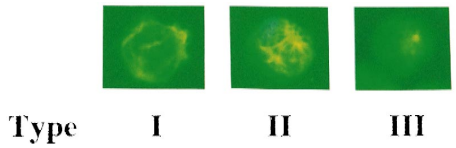

Fig. 2. Representative photomicrographs of abnormal spindles. Under the conditions we used, type IV spindles were not seen.

min at room temperature, and treated with 0.3% Triton X-100 for 10 min at 37°C. The cells were then incubated with 10 ng/ml fluorescein isothiocyanate for 1 h at room temperature, pelleted, resuspended in phosphate-buffered saline containing RNase A and propidium iodide, and analyzed by flow cytometry.

2.6. Assay of relative immunofluorescence intensities by flow cytometry

The cyclin B, cdc2, cyclin E, cdk2 and tubulin proteins were detected by indirect immunofluorescence using specific antibodies and standard methodology (Benito et al., 1995). Meth-A cells were collected, washed in phosphate-buffered saline, fixed with 1% paraformaldehyde for 30 min at room temperature, and treated with 0.3% Triton X-100 for 10 min at 37°C. The cells were then incubated with the primary rabbit polyclonal antibodies against cyclin B, cdc2 or cyclin E, or primary mouse monoclonal antibodies against cdk2 or tubulin, for 24 h at 4°C. The cells were washed in phosphate-buffered saline containing 1% bovine serum albumin and stained with the secondary goat anti-rabbit (cyclin B, cdc2, and cyclin E) or rabbit anti-mouse (cdk2 and tubulin) immunoglobulin G (IgG) fluorescein isothiocyanate-conjugated antibodies. The cells were then washed again, resuspended in phosphate-buffered saline containing RNase A and propidium iodide, and analyzed by flow cytometry.

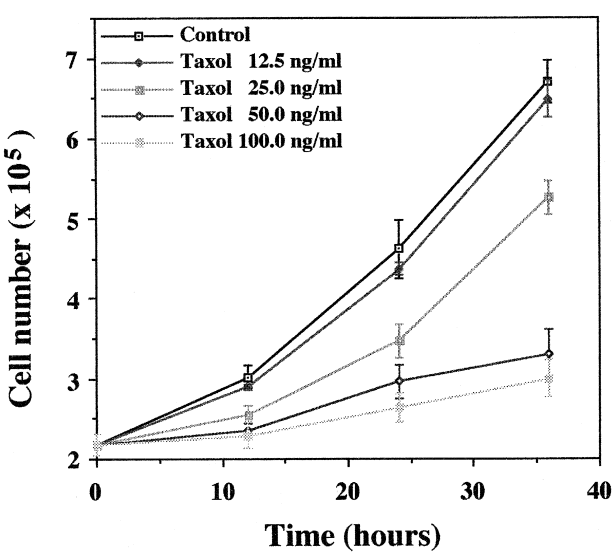

Fig. 3. Taxol inhibits proliferation of Meth-A cells. Meth-A cells were treated with 0, 12.5, 25, 50 or 100 ng/ml taxol for 36 h. The number of cells was determined using a Coulter Counter.

2.7. Western blot analysis of proteins

Western blot analysis of the expression of cdc2 and cdk2 proteins was performed using specific antibodies and

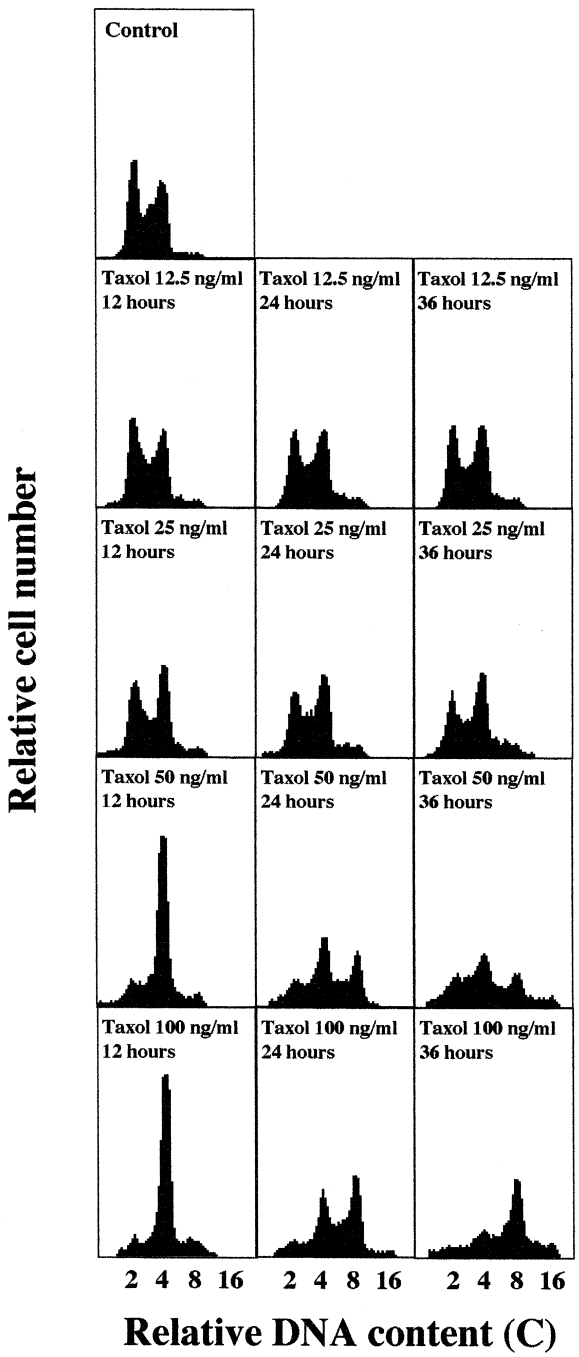

Fig. 4. Taxol induces polyploidization of Meth-A cells. Meth-A cells were treated with 0, 12.5, 25, 50 or 100 ng/ml taxol for 36 h. The cells were collected and the distribution of DNA content was determined by flow cytometry at 0, 12, 24 and 36 h, respectively. C: haploid DNA content.

standard methodology (Banerjee et al., 1998). Briefly, protein was extracted from the cells with lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 μg/ml aprotinin, 1% NP-40, and 1 mM phenylmethylsalfonylfluoride. The lysate supernatant was harvested by centrifugation at 15,000 × *g* for 30 min. Appropriate protein amounts (20 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto a nitrocellulose membrane. The membranes were probed with the respective antibody and then with antirabbit or antimouse peroxidase-conjugated secondary IgG antibodies.

2.8. Analyses of morphological changes

For immunohistochemistry of tubulin protein from Meth-A cells, cells were stained as for flow cytometry and observed using fluorescence microscopy.

The abnormal spindles were classified according to the increase in disorganization as follows: metaphase spindles of control cells (the spindles were bipolar with very few or no detectable astral microtubules), type I spindles (the astral microtubules were more prominent, longer, and more numerous than those of normal spindles), type II spindles (the spindles were still bipolar, but the spindle clearly showed some signs of collapse and the astral microtubules were longer than type I spindles), type III spindles (the spindles were significantly collapsed, appeared monopolar, and consisted of one or more star-shaped aggregates of microtubules), and type IV spindles (microtubules were virtually non-existent) (Jordan et al., 1992). Between 50 and several hundreds of metaphases were scored for each group to determine the frequency of normal and types I–IV spindles (Fig. 2).

3. Results

3.1. Taxol inhibits proliferation of Meth-A cells

For this study, exponentially growing Meth-A cells in non-synchronized cultures were treated with 0, 12.5, 25, 50 or 100 ng/ml of taxol and then cultivated for 36 h. Cells were counted 0, 12, 24 and 36 h after taxol addition. Fig. 3 shows the proliferation curves of the cells as a function of time of taxol treatment. The inhibition of proliferation of Meth-A cells by taxol was clearly observed in concentration- and time-dependent manners during the 36-h period.

3.2. Taxol induces polyploidization of Meth-A cells

To investigate the mechanism by which taxol inhibits the proliferation of Meth-A cells, we examined the effects

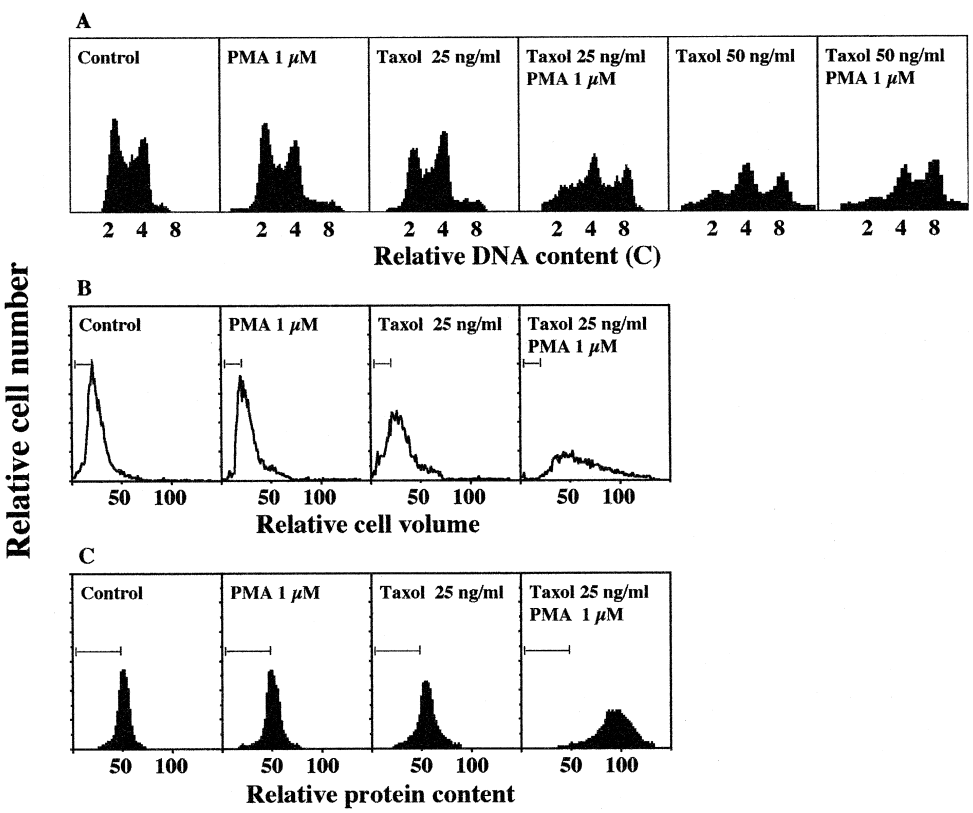

Fig. 5. Effects of taxol in the presence of PMA on cell cycle, cell volume and protein content. Meth-A cells were treated with PMA, taxol, or taxol in the presence of PMA for 24 h. The cells were collected, and the distribution of DNA content (A), cell volume (B), and protein content (C) was determined. C: haploid DNA content.

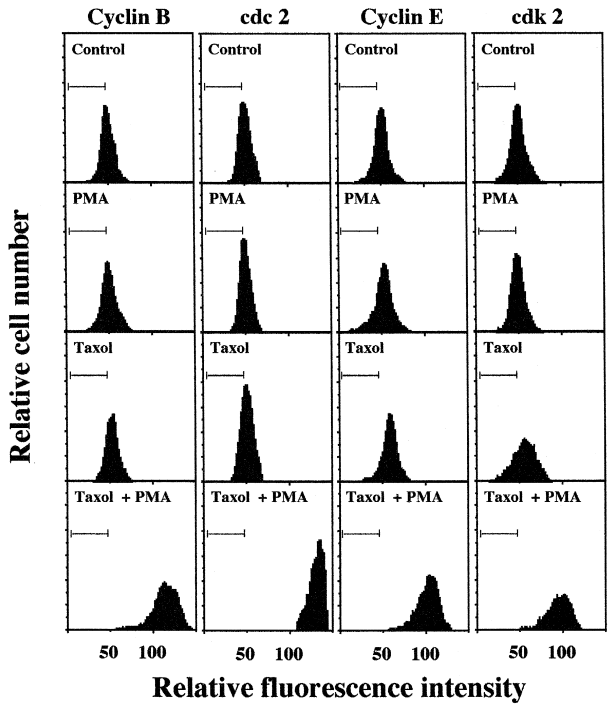

Fig. 6. Effects of taxol in the presence of PMA on G1 and G2 checkpoints. Meth-A cells were treated with 25 ng/ml taxol, 1 μM PMA, or 25 ng/ml taxol in the presence of 1 μM PMA for 24 h. The cells were fixed with 1% paraformaldehyde and treated with 0.3% Triton X-100, and then incubated with primary antibodies against cyclin B, cdc2, cyclin E, or cdk2. The cells were washed in phosphate-buffered saline containing 1% bovine serum albumin and stained with secondary fluorescein isothiocyanate-conjugated anti-IgG antibodies. The cells were then washed again, resuspended in phosphate-buffered saline containing RNase A and propidium iodide, and analyzed by bivariate flow cytometry.

of taxol on the cell cycle. The cells were stained with propidium iodide and subjected to flow cytometry in order to examine the distribution of DNA content. The resultant histograms are shown in Fig. 4. The first peak was produced by cells with a 2C DNA content, the second with a 4C, the third with an 8C, and the fourth with a 16C DNA content.

Exposure to 12.5 ng/ml taxol for 12 h did not obviously affect the distribution of DNA content. With increasing concentrations of taxol, the main peak shifted from 2C in the control to 4C at 12 h. At 100 ng/ml, in most of the cells DNA was accumulated in the 4C peak, with small 2C and 8C peaks. With longer incubation in the presence of taxol, cells with progressively greater DNA contents were observed until 36 h. After 36 h in the presence of 100 ng/ml taxol, the main peak was further shifted to 8C and a 16C peak appeared.

3.3. Effects of taxol in the presence of PMA on cell cycle, cell volume and protein content

PMA was used to examine the role of protein kinase C in polyploid formation. As shown in Fig. 5, treatment with 25 ng/ml taxol for 24 h increased the proportion of cells with a 4C DNA content, slightly enlarged the cell volume, and slightly increased the protein content. An amount of 1 μM PMA alone did not significantly affect these parameters. However, in the presence of 1 μM PMA, 25 ng/ml taxol clearly induced the appearance of an 8C peak, similar to that induced by treatment with 50 ng/ml taxol for 24 h. Similarly, taxol plus PMA more markedly increased the cell volume and the protein content than did taxol alone.

3.4. Effects of taxol in the presence of PMA on G1 and G2 checkpoints

The fact that treatment with 25 ng/ml taxol in the presence of 1 μM PMA for 24 h induced polyploidization implies that, under these conditions, the progression of the cell cycle was accelerated, while the metaphase/anaphase transition was blocked. To clarify the role of the G1 and G2 checkpoints in the induction of polyploidization, flow cytometry was used to quantify the expression of cyclin E and cdk2 proteins for the G1 checkpoint, and cyclin B and cdc2 proteins for the G2 checkpoint.

Fig. 6 shows the expression of cyclin E, cdk2, cyclin B, and cdc2 proteins in Meth-A cells subjected to flow cytometry. After exposure to 25 ng/ml taxol, 1 μM PMA, or 25 ng/ml taxol in the presence of 1 μM PMA for 24 h, the expression patterns of the four proteins were similar. Namely, PMA itself did not significantly affect the expression of these proteins, while taxol slightly increased their expression, and taxol in the presence of PMA increased their expression more markedly. Similar results for cdc2 and cdk2 expression were observed by Western blot analysis (Fig. 7).

The overexpression of these proteins may cause the acceleration of the progression of the cell cycle and induce polyploidization.

3.5. Effects of taxol in the presence of PMA on tubulin content and spindle organization

After 24 h of exposure to 1 μM PMA, 25 ng/ml taxol, or 25 ng/ml taxol in the presence of 1 μM PMA, tubulin was quantified using flow cytometry. It is interesting that taxol clearly increased the expression of tubulin, although taxol did not induce polyploidization at this concentration. Although PMA itself slightly increased the expression of

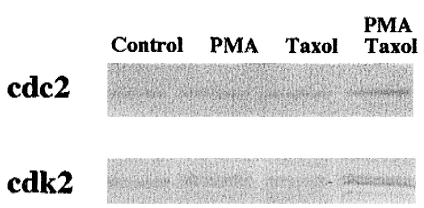

Fig. 7. Analysis of cdc2 and cdk2 expression by Western blotting. The cells were treated as described in the legend to Fig. 6 and the expression of cdc2 and cdk2 was detected by Western blot analysis as described in Section 2.

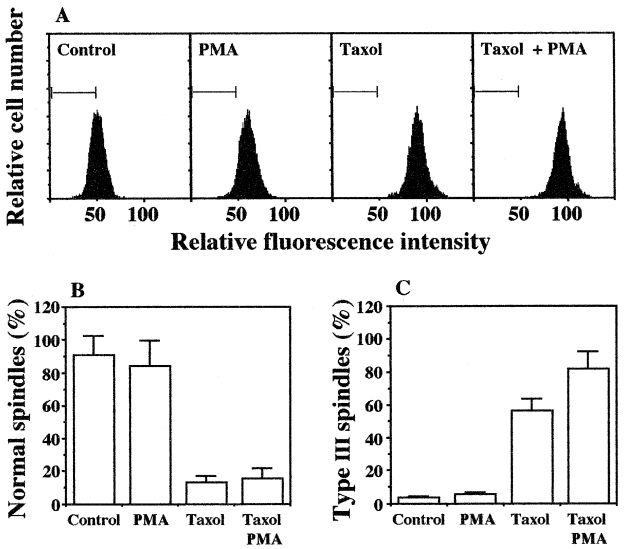

Fig. 8. Effects of taxol in the presence of PMA on tubulin content and spindle organization. Meth-A cells were treated with 25 ng/ml taxol, 1 μM PMA, or 25 ng/ml taxol in the presence of 1 μM PMA for 24 h. The cells were treated as described in the legend of Fig. 6, and the content of tubulin protein was then analyzed by bivariate flow cytometry (A) and spindle organization was observed by fluorescence microscopy (B, C). The abnormal spindles were classified into four types as described in Section 2.

tubulin, PMA did not potentiate the effect of taxol on tubulin expression (Fig. 8A).

To examine the effect of taxol, especially in the presence of PMA, on spindle organization, the cells used to quantify tubulin expression were observed by fluorescence microscopy. Disorganization of the spindle was obvious after treatment with 25 ng/ml taxol (Fig. 8B). PMA had no obvious effect on spindle organization itself or on taxol-induced spindle disorganization. However, when we classified the abnormal spindles into four types according to the degree of disorganization, we found that while more than 50% of spindles were type III after taxol treatment, in the presence of PMA plus taxol, more than 80% of the spindles were type III (Fig. 8C).

4. Discussion

A number of experiments have demonstrated that protein kinase C is closely associated with the pharmacological effects of taxol. Jun et al. (1995) found that protein kinase C is involved in taxol-induced activation of murine peritoneal macrophages. Wang et al. (1998a) obtained evidence suggesting that bryostatin 1, an activator of protein kinase C, potentiated taxol-induced apoptosis. In the present study, polyploidization of Meth-A cells was caused by the addition of taxol to the medium. To examine the relationship between the induction of polyploidization and protein kinase C, taxol in the presence of PMA was used to treat Meth-A cells. We found that not only taxol-induced polyploidization was potentiated by PMA, but also that polyploidization was induced by a lower concentration (25 ng/ml) of taxol, which did not induce polyploidization by itself, in the presence of PMA. These findings indicated that protein kinase C is involved in the induction of polyploidization.

The fact that 25 ng/ml taxol did not induce polyploidization by itself, but did in the presence of PMA, implies that PMA accelerated the progression of the cell cycle in the absence of mitosis. To examine the mechanism, by which PMA accelerates the progression of the cell cycle during induction of polyploidization, the checkpoints in the G1/S and G2/M transitions were investigated. We measured the G1 checkpoint-related proteins cyclin E and cdk2, and G2 checkpoint-related proteins cyclin B and cdc2 using flow cytometry, and found that the amount of both G1 and G2 checkpoint-related proteins increased during the induction of polyploidization. These findings raised the possibility that protein kinase C is associated with up-regulation of G1 and G2 checkpoint-related proteins, which are determinants of the induction of polyploidization although kinase activities are also important. These findings are consistent with evidence demonstrating a functional link between protein kinase C and cell cycle checkpoints, and a number of specific effects caused by different members of the protein kinase C family has been reported (Garcia and Cales, 1996; Zeng and el-Deiry, 1996; Arita et al., 1998).

Ball et al. (1992) reported that PMA increases tubulin synthesis and stimulates tubulin polymerization. Lehrich and Forrest (1994) and Garcia-Rocha et al. (1997) presented direct evidence that protein kinase C binds to tubulin. These observations suggest that tubulin may play a central role in the polyploidization induced by taxol in the presence of PMA. Our results obtained by flow cytometry showed that taxol treatment increased the total content of tubulin protein, and PMA did not influence this effect of taxol. However, morphological data showed that PMA potentiated the induction of abnormal spindles by taxol. It is clear that protein kinase C is involved in taxol-induced polyploidization through interference with tubulin polymerization. Studies to gain further insight into the mechanism by which protein kinase C is associated with the induction of polyploidization are in progress.

Taken together, the findings show that, in this cell line, PMA potentiates taxol-induced polyploidization in at least two ways, i.e., accelerating the progression of the cell cycle by up-regulation of G1 and G2 checkpoint-related proteins and potentiating spindle disorganization which blocks mitosis. These findings suggest that appropriate drug combinations with taxol may be clinically useful.

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