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Taxol Induces Concomitant Hyperphosphorylation and Reorganization of Vimentin Intermediate Filaments in 9L Rat Brain Tumor Cells

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Abstract Taxol, a microtubule stabilizing agent, has been extensively investigated for its antitumor activity. The cytotoxic effect of taxol is generally attributed to its antimicrotubule activity and is believed to be cell cycle dependent. Herein, we report that taxol induces hyperphosphorylation and reorganization of the vimentin intermediate filament in 9L rat brain tumor cells, in concentration- and time-dependent manner. Phosphorylation of vimentin was maximum at 10^{-6} M of taxol treatment for 8 h and diminished at higher (10^{-5} M) concentration. Enhanced phosphorylation of vimentin was detectable at 2 h treatment with 10^{-6} M taxol and was maximum after 12 h of treatment. Taxol-induced phosphorylation of vimentin was largely abolished in cells pretreated with staurosporine and bisindolymaleimide but was unaffected by H-89, KT-5926, SB203580, genistein, and olomoucine. Thus, protein kinase C may be involved in this process. Hyperphosphorylation of vimentin was accompanied by rounding up of cells as revealed by scanning electron microscopy. Moreover, there was a concomitant reorganization of the vimentin intermediate filament in the taxol-treated cells, whereas the microtubules and the actin microfilaments were less affected. Taken together, our data demonstrate that taxol induces hyperphosphorylation of vimentin with concomitant reorganization of the vimentin intermediate filament and that this process may be mediated via a protein kinase C signaling pathway. J. Cell Biochem. 68:472–483, 1998.

Key words: taxol; microtubules; vimentin; intermediate filaments; protein phosphorylation; protein kinases; inhibitors; cytoskeleton

Taxol is a diterpenoid plant product [Wani et al., 1991] that has been used as an antitumor drug for the treatment of ovarian and breast carcinomas [Seidman et al., 1997; Dunton, 1997]. The drug enhances tubulin polymerization, stabilizes microtubules (MTs), and prevents MT depolymerization induced by calcium or low temperature [Schiff and Horwitz, 1980; 1981; Thompson et al., 1981; Horwitz, 1992]. The unusual stability of MTs in taxol-treated cells leads to mitotic block resulting in inhibition of cell division [Jordan et al., 1993; Long and Fairchild, 1994]. It is generally believed that taxol exerts its cellular activity by binding directly to MTs [Horwitz, 1992], and several taxol binding sites on MTs have been proposed [Rao et al., 1994; 1995; Nogales, 1995]. However, the effectiveness of taxol in blocking mitosis and its antitumor activity exceed that of other MT targeting drugs, suggesting that it may have additional modes of action [see Lee et al., 1996 and references therein].

Microtubules, intermediate filaments (IFs), and actin microfilaments (AFs), are the major constituents of cytoskeleton in typical mammalian cell. In conjunction with associated proteins, these filamentous networks are considered to establish and maintain cellular organization and perform vital functions in differentiation and development [Bershadsky and Vasiliev, 1988; Kelly, 1990; Hyams and Lloyd, 1994]. MTs are mainly composed of tubulin

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subunits, along with a cohort of MT-associated proteins [Hyams and Lloyd, 1994], whereas IFs are formed by various polypeptides grouped into six types, on the basis of amino acid sequence homologies and selective expression in different cell types [Stewart, 1993; Fuchs and Weber, 1994]. In cells of mesenchymal origin and most cells in culture, the most abundant IF polypeptide is vimentin, which is organized in a filamentous meshwork connecting the nuclear envelope and the plasma membrane [Skalli and Goldman, 1991; Maniotis et al., 1997]. It has been repeatedly suggested that vimentin IFs and MTs are closely associated [Draberova and Draber, 1993; Gurland and Gundersen, 1995; Trevor et al., 1995; Svitkina et al., 1996]; thus, disturbance of MTs may also affect the organization of IFs. For instance, cells treated with MT depolymerizing agents, such as colchicine, vincristine, vinblastine, and nocodazole, lead to perinuclear collapse of the IF networks [Hollenbeck et al., 1989].

Reorganization of vimentin IFs is always accompanied by increase in phosphorylation of the vimentin molecules [Gard and Lazarides, 1982; Sternberger and Sternberger, 1983; Escribano and Rozengurt, 1988; Lee et al., 1992; 1993; Cheng and Lai, 1994]. For instance, the dynamic change in IF organization during mitosis is demonstrated to be mediated by cdc2 kinase and protein kinase C (PKC) [Chou et al., 1990; Matsuoka et al., 1994; Tsujimura et al., 1994; Takai et al., 1996]. However, the protein kinase(s) responsible for the phosphorylation of vimentin were not always identified in stimulated interphase cells. On the other hand, treatment with protein kinase activators or protein phosphatase inhibitors results in vimentin phosphorylation and reorganization [Evans, 1988; Harrison and Mobley, 1991; Lee et al., 1992; Eriksson et al., 1992; Ho and Boberge, 1997]. Therefore, it is now well established that increase in vimentin phosphorylation leads to reorganization of vimentin IF, and vice versa.

Vimentin has been shown to be a substrate for a large array of protein kinases. For instance, vimentin has been demonstrated to be phosphorylated by cAMP-dependent protein kinase (PKA) [O'Conner et al., 1981; Ando et al., 1989; Lamb et al., 1989], PKC [Huang et al., 1988; Ando et al., 1989], cGMP-dependent protein kinase (PKG) [Wyatt et al., 1991], Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) [Ando et al., 1991], p34cdc2 kinase [Chou et al., 1990; 1991], autophosphorylation-dependent protein kinase [Huang et al., 1994], p37 protein kinase [Chou et al., 1996], and PNK, a serine/threonine protein kinase with a catalytic domain homologous to the PKC family and unique N-terminal leucine zipper-like sequences [Matsuzawa et al., 1997]. A total of more than 15 serine/threonine residues are identified as potential phosphorylation sites on this protein [Huang et al., 1994]. Despite the presence of so many potential phosphorylation sites, only a small fraction of vimentin is phosphorylated under normal conditions [Cabral and Gottesman, 1979, Nelson and Traub, 1983, Lee et al., 1992; Lai et al., 1993; Cheng and Lai, 1994]. By contrast, rapid increase in vimentin phosphorylation has been reported in cells during mitosis [Foisner, 1997], or treatment with hormones [Spruill et al., 1983], cAMP [Gard and Lazarides, 1982; Escribano and Rozengurt, 1988], heat shock [Cheng and Lai, 1993], withangulatin A [Lee et al., 1993], okadaic acid [Yatsunami et al., 1991; Lee et al., 1992; Lai et al., 1994], and fostriecin [Ho and Roberge, 1997]. In response to these treatments, vimentin IF switches from an extended filamentous form to a higher-order organization. Although the functions of IF protein remain to be fully elucidated, several regulatory roles of IF protein phosphorylation have been identified or are emerging. These roles include filament disassembly and reorganization, solubility, localization within specific cellular domains, association with other cytoplasmic or membrane-associated proteins, protection against physiological stress, and mediation of tissue-specific functions, among others [see Ku et al., 1996].

Based on the fact that MT poisons will induce reorganization of vimentin IFs and that reorganization of IFs is always accompanied by phosphorylation of the IF proteins, concomitant phosphorylation and reorganization of vimentin IFs induced by MT perturbing drugs can be expected. There have been relatively few studies investigating the alterations in IF phosphorylation and structure during taxol-induced cell injury. The present study investigates the alterations of vimentin phosphorylation and IF organization in taxol-treated 9L rat brain tumor cells (RBT cells). Vimentin has been shown as the major IF component to be phosphorylated and reorganized in cells subjected to a variety of external stimuli [Lee et al., 1992; 1993; Lai et al., 1993; Cheng and Lai, 1994; Chen et al., 1996]. In this communication, we demonstrate

that vimentin IFs are hyperphosphorylated in cells treated with taxol and the effect is both concentration and time dependent. We also show that taxol-induced phosphorylation of vimentin may be mediated by PKC. Furthermore, concomitant alterations in cell shape as well as cytoskeletal architecture were observed.

MATERIALS AND METHODS

Materials

Culture medium components were purchased from Gibco Laboratories (Grand Island, NY) and cultureware was obtained from Corning (Corning, NY). [^{32}P]orthophosphate (spec act 8,500–9,120 Ci/mmole) was from New England Nuclear (Boston, MA). Chemicals for electrophoresis were purchased from BioRad (Richmond, CA). Mouse monoclonal antibodies against β-tubulin, alkaline phosphatase- and rhodamine-conjugated goat anti-mouse IgG were purchased from Boehringer Mannheim (Postfach, Germany). Mouse monoclonal antibodies against vimentin were obtained from Amersham (Little Chalfont, UK). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). Protein kinase inhibitors, including H-89, KT-5926, BIM, staurosporine, genistein, and olomoucine, were obtained from Calbiochem (La Jolla, CA). SB203580 was a kind gift from Smithkline Beecham Pharmaceuticals (King of Russia, PA). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture, Drug Treatment, and Metabolic Labeling

9L RBT cells [Weizsaeker et al., 1981] were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C under a 5% CO_2 atmosphere. Exponentially growing cells at 80–90% confluency were used for all experiments. Taxol was dissolved in ethanol at a stock concentration of 1 mM and stored at −20°C in the dark. For drug treatment, the stock solution was added to the desired final concentration and the cells were treated for various durations, as indicated. Phosphorylation of intracellular proteins in taxol-treated cells was revealed by metabolic labeling with [^{32}P]orthophosphate. Cells were labeled with 1 mCi/ml of [^{32}P]orthophosphate for 2 h before harvesting in all treatment protocols. To study the effects of protein kinase inhibitors on protein phosphorylation, in taxol-treated cells, the cells were pre-incubated with 5×10^{-6} M H-89, 5×10^{-8} M KT-5926 (both for PKA), 2×10^{-7} M BIM (PKC), 10^{-8} M staurosporine (general protein kinases), 4×10^{-6} M SB203580 (p38 MAPK), 10^{-6} M genistein (tyrosine kinases), and 5×10^{-3} M olomoucine (cdc2 kinase) for 6 h prior to taxol treatment and metabolic labeling.

SDS-PAGE, Autoradiography, and Immunoblotting

After treatment and labeling, the cells were washed with phosphate-buffered saline (PBS) and lysed in sample buffer [Laemmli, 1970]. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970]. Sample preparation and other related experimental procedures were carried out as previously described [Lai et al., 1993]. Bands of interest on the autoradiographs were quantified by densitometric scanning in 2-D mode (Molecular Dynamics, San Francisco, CA). Alternatively, the gels were soaked in transfer buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) for 10 min, and the proteins were electrotransferred onto nitrocellulose membranes (Hybond-C Extra, Amersham) by a semidry method (OWL Scientific Plastic, Cambridge, MA). The membranes were incubated for 1 h with 3% gelatin in Tween 20 containing Tris-buffer saline (TTBS: 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS briefly. Subsequently, the membranes were incubated with monoclonal antibodies to vimentin (diluted 1:200 in TTBS containing 1% gelatin) at room temperature for 2 h. After three washes with TTBS, immune complexes on the membranes were reacted with alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1:2,000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membranes were then rinsed three times with TTBS and dried. The immunoblots were developed, at room temperature, in developing buffer (15 mg of nitro blue tetrazolium, 0.7% N,N-dimethylformamide, 30 mg of 5-bromo-4-chloro-3-indolyl

phosphate per dl 100 mM $NaHCO_3$, at pH 9.8, containing 1 mM $MgCl_2$).

Scanning Electron Microscopy

For scanning electron microscopy, cells on coverslips were fixed directly in 2.5% glutaraldehyde solution containing 4.5% glucose, buffered with 75 mM cacodylate (pH 7.2), at 4°C for 40 min. After washing with 100 mM cacodylate buffer three times, cells were postfixed in 1% osmium tetroxide buffered with 50 mM cacodylate (pH 7.2), at 4°C for 40 min. Subsequently, cells were washed with milli-Q water, dehydrated in an ethanol series, critical-point dried, mounted on a stub, coated with gold–palladium, and examined by a Hitachi S-2300 SEM at 15 kV.

Fluorescence Microscopy for Actin, Tubulin, and Vimentin

Actin were fluorescent-labeled by rhodamine-conjugated phalloidin. After rinsing in PBS, the cells were fixed in 4% paraformaldehyde at 4°C for 20 min, permeabilized with 0.05% Triton X-100 at room temperature for 10 min, and then labeled with rhodamine-conjugated phalloidin (diluted 1:20) for 20 min. All fluorescence staining procedures were performed in the dark. MTs and vimentin IFs were visualized by indirect immunofluorescence microscopy. All procedures were carried out at room temperature; cell fixing was done at 4°C. Blocking of nonspecific sites and dilution of antibodies were done in 1% bovine serum albumin (BSA) in PBS. The fixed cells were rinsed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After a further wash in PBS, cells were incubated in blocking buffer for 1 h and then incubated with mouse monoclonal antibody against tubulin (diluted 1:40) or vimentin (diluted 1:50). The cells were then washed extensively with PBS, blocked again for 30 min and allowed to react with rhodamine-conjugated goat anti-mouse IgG (diluted 1:50) for 1 h. After a final extensive wash with PBS, the cells were mounted in anti-bleach mounting fluid, 5% propylgallate dissolved in 15% PBS/85% glycerol, and stored in the dark at 4°C until viewed. The specimens were examined under a Zeiss Axiophot microscope equipped with epifluorescence optics and all images were recorded using a Plan-neofluar 100× (N.A. 1.4) objective.

RESULTS

Effects of Taxol on Vimentin Phosphorylation in 9L RBT Cells

To investigate whether taxol affects protein phosphorylation, cells were treated with 10^{-6} M taxol for 6 h and metabolically labeled with [^{32}P]orthophosphate during the last 2 h of drug treatment. In parallel experiments, cells were also heat shocked or treated with OA. The phosphorylation level of a 57-kDa protein was significantly enhanced in the taxol-treated cells (Fig. 1A). This protein was identified as vimentin by comparison with the heat shocked and OA-treated samples as well as by Western blotting (Fig. 1C). Furthermore, the protein level was unaltered by the treatments as indicated by Coomassie blue and the Western blotting patterns (Fig. 1B,C). Interestingly, the phosphorylation of vimentin was much more profound in OA-treated cells, and this phosphovimentin exhibited slightly lower mobility in gel electrophoresis (Fig. 1, lanes 4, 8, and 12), suggesting that the protein kinase(s) involved may be different in these samples.

Dose-Dependent Effects of Taxol on Phosphorylation of Vimentin and Other Alkaline-Stable Phosphoproteins in 9L RBT Cells

To assess the kinetics of vimentin phosphorylation in taxol-treated samples, 9L cells were treated with various concentration of taxol for different periods and the phosphorylation levels of vimentin were monitored by metabolic labeling with [^{32}P]orthophosphate. The phosphorylation of vimentin was detectable in cells treated for 8 h with 10^{-8} M taxol (about 2.23-fold as compared to the untreated controls) and was significantly enhanced by 10^{-6} M taxol (about 7.55-fold) (Fig. 2A; see Fig. 4). At higher concentration of 10^{-5} M taxol, however, the phosphorylation completely diminished; phosphorylation of other proteins remained largely unaffected (Fig. 2A, lane 5). These data indicate that a specific taxol-sensitive protein kinase is involved in this process. Taxol-induced phosphorylation of vimentin was time dependent. Upon treatment with 10^{-6} M taxol for various periods, phosphorylation of vimentin was unaffected during the first 2 h, and it gradually enhanced after 4 h of treatment (Fig. 3A). The phosphorylation level of vimentin increased by 7.55-, 9.46- and 10.46-fold after 8, 10, and 12 h, respectively (Fig. 4). In order to reveal the

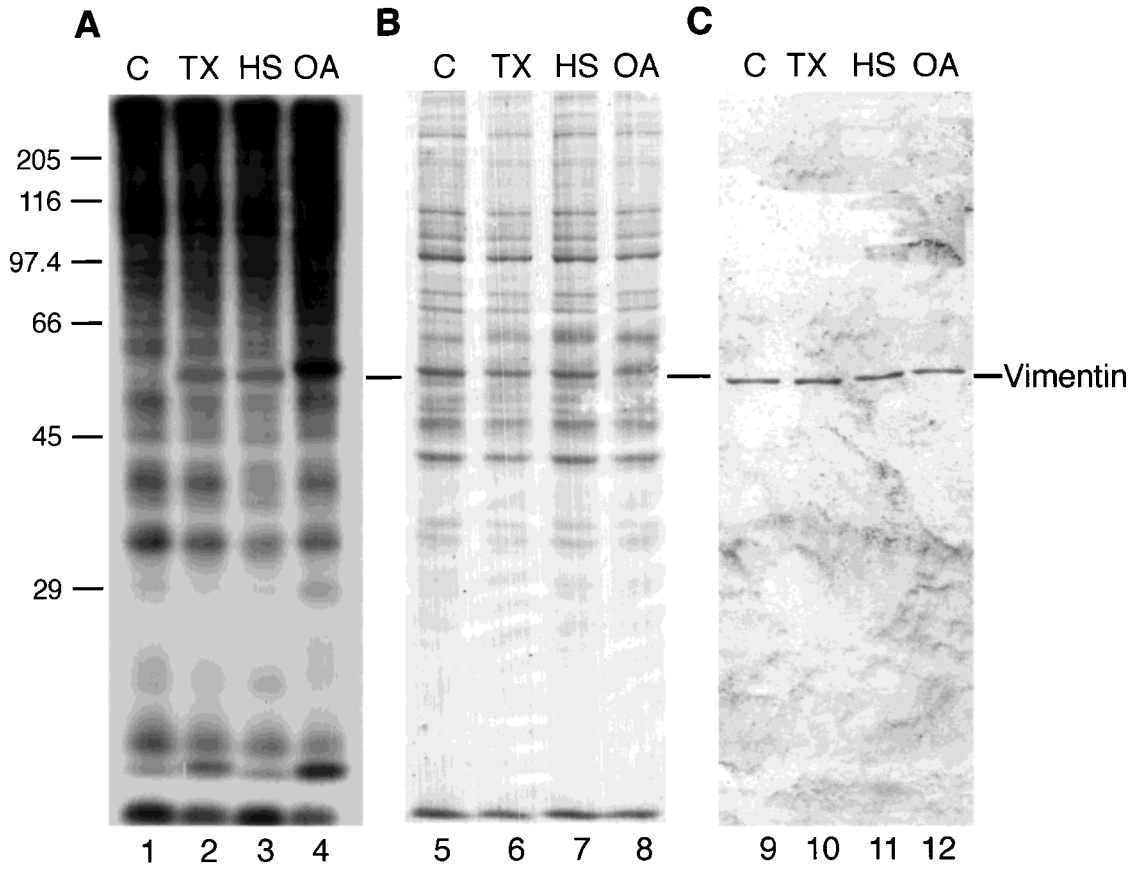

Fig. 1. Effect of taxol on vimentin phosphorylation in 9L RBT cells. Cells were treated with 10^{-6} M taxol for 6 h and metabolically labeled with [^{32}P]orthophosphate (1 mCi/ml) during the last 2 h of drug treatment (TX). Alternatively, cells were heat shocked at 45°C for 30 min (HS), or treated with 3×10^{-7} M okadaic acid for 2 h (OA). Metabolic labeling with [^{32}P]orthophosphate (1 mCi/ml) in heat-shocked or OA-treated cells was done in a way that all cells were labeled for 2 h just before harvesting. After treatment, the cells were lysed with SDS sample buffer and the proteins were resolved by 10% SDS-PAGE. After electrophoresis, the gels were processed for autoradiography **(A)** or stained with Coomassie blue **(B).** In parallel experiments, the polypeptides were electrotransferred onto a nitrocellulose membrane and then processed for immunoblotting by using monoclonal antibody to vimentin as a probe **(C).** Molecular weight (in kDa) standards are shown at the left. **Lanes 1, 5, 9** (C), Untreated controls; **lanes 2, 6, 10** (TX); taxol-treated cells; **lanes 3, 7, 11** (HS), heat-shocked cells; **lanes 4, 8, 12** (OA); OA-treated cells.

changes in phosphorylation levels of alkaline-stable phosphoproteins, duplicate gels were treated with KOH prior to autoradiography. The results show that the phosphorylation levels of two phosphoproteins with molecular weights of 106 and 36 kDa (pp106 and pp36, respectively) were enhanced in a dose-dependent manner, while that of pp65 remained almost unaffected (Figs. 2B, 3B, and 4).

Effects of Protein Kinase Inhibitors on Taxol-Induced Phosphorylation of Vimentin

As a first step to identify the protein kinase involved in taxol-induced phosphorylation of vimentin, a number of specific protein kinase inhibitors were used in conjunction with taxol treatment. The concentration used for each inhibitors was pretested to assure that the cell viability was not affected (data not shown). It was found that phosphorylation of vimentin was largely abolished upon preincubation of the cells with 10 nM staurosporine (a general protein kinase inhibitor) or 200 nM BIM (a specific PKC inhibitor) (Fig. 5A,B). However, pretreatment of the cells with 5 μM H-89, 50 nM KT-5926 (both specific PKA inhibitors), 4 μM SB203580 (p38 MAPK inhibitor), 1 μM genistein (inhibitor for tyrosine kinases), or 5 mM olomoucine (cdc2 kinase specific inhibitor), had a negligible effect on taxol-induced phosphorylation of vimentin (Fig. 5A,B). These results point to a role of PKC in taxol-induced

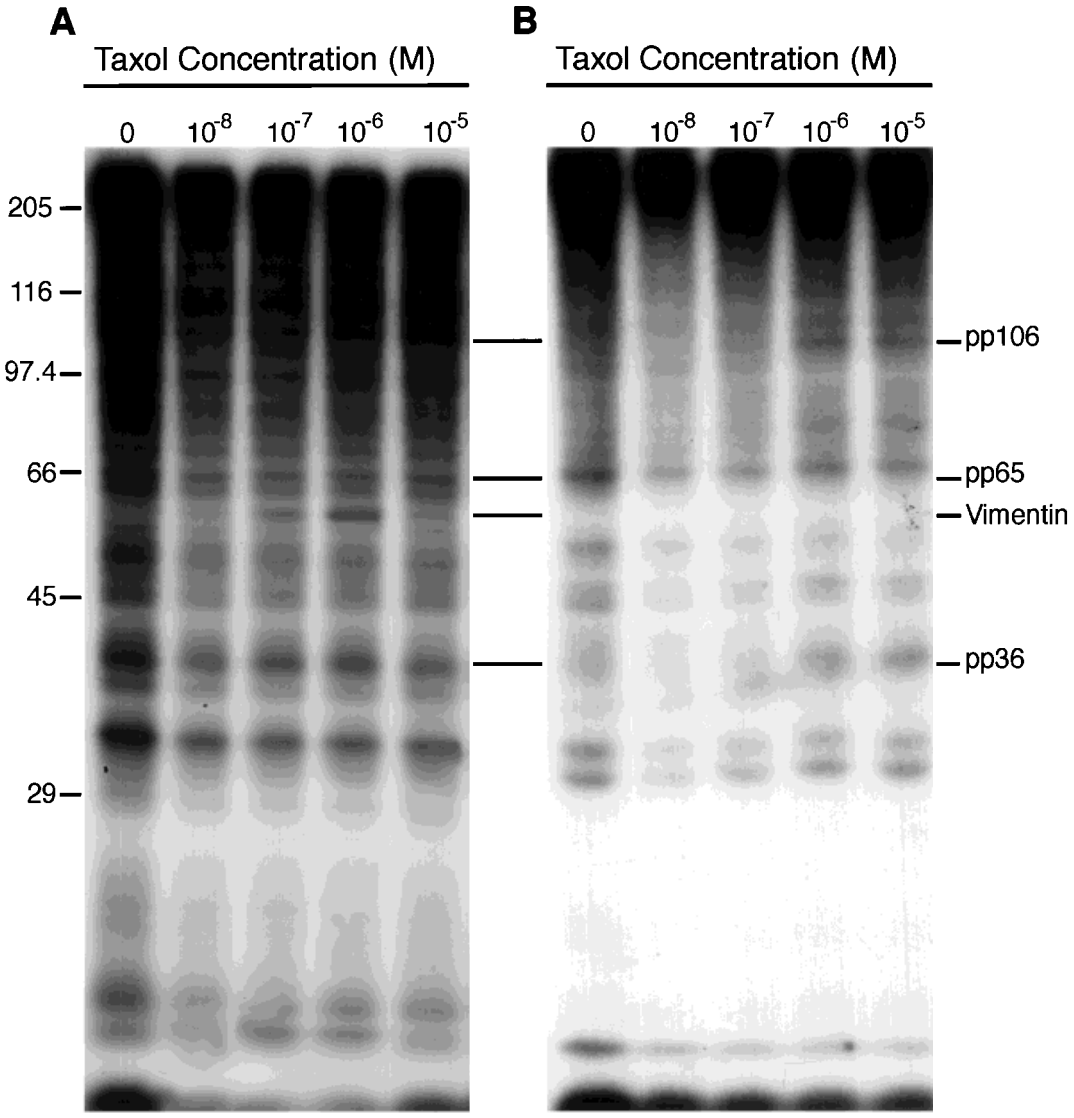

Fig. 2. Concentration-dependent effects of taxol on protein phosphorylation in 9L RBT cells. Cells were incubated with indicated taxol concentrations for 8 h and were labeled with [^{32}P]orthophosphate for 2 h prior to harvesting. After treatment, the cells were lysed with SDS sample buffer and the proteins were resolved by 10% SDS-PAGE. After electrophoresis, the gels were processed for autoradiography **(A).** Alternatively, the gels were heated in 1 M KOH at 55°C for 2 h before processing for autoradiography **(B).** Molecular weight (in kDa) standards are shown at the left. Vimentin as well as alkaline-stable phosphoproteins with molecular weights of 106, 65, and 36 kDa (i.e., pp106, pp65, and pp36, respectively) are marked at the right.

enhancement of vimentin phosphorylation in 9L cells.

Effects of Taxol on Morphology of 9L RBT Cells

The morphology and cytoskeletal organization in the taxol-treated cells were monitored by scanning electron microscopy and fluorescence microscopy, respectively. In the presence of taxol, the number of rounded up cells (resembling those in the mitotic phase) continued to increase with length of treatment time. Upon treating the cells with 10^{-6} M taxol, the morphological change was detectable after 2 h and was obvious after 4 h of treatment. At the end of 8 h, a large portion of the cells rounded up on the substratum and became spherical (Fig. 6C). Since cell morphology is apparently maintained by the cytoskeleton, the plausible reorganization of the cytoskeletal networks was explored by using fluorescence microscopy. It was found that after 4 h of treatment with 10^{-6} M taxol, the MTs and MFs retracted slightly from

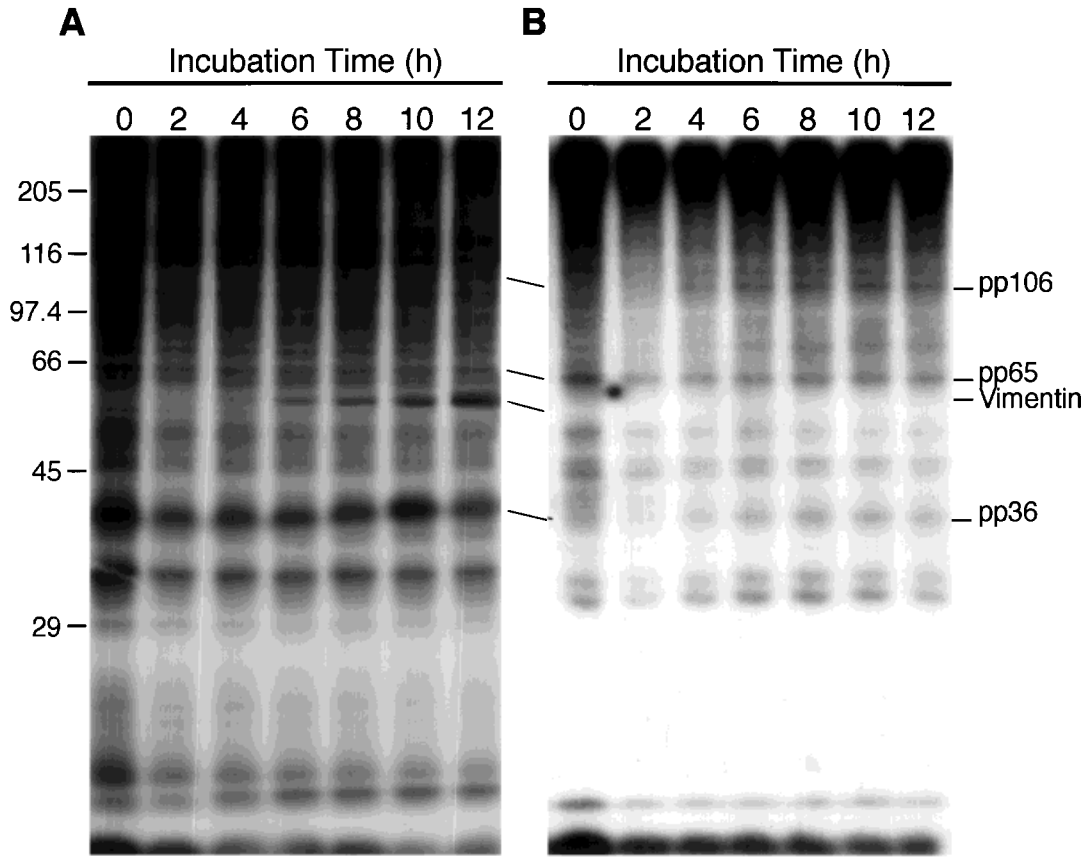

Fig. 3. Time-dependent effects of taxol on protein phosphorylation in 9L RBT cells. Cells were treated with 10^{-6} M taxol for up to 12 h and were labeled with [^{32}P]orthophosphate for 2 h just before harvesting at 2 h intervals. Subsequent experimental procedures were same as previous experiments. **A:** Autoradiograph of the phosphoproteins in taxol-treated 9L RBT cells. **B:** Autoradiograph of alkaline-stable phosphoproteins in taxol-treated 9L RBT cells. Molecular weight (in kDa) standards are shown at the left. Vimentin as well as alkaline-stable phosphoproteins with molecular weights of 106, 65, and 36 kDa (i.e., pp106, pp65, and pp36, respectively) are marked at the right.

the cell periphery (Fig. 7D,E), whereas the retraction of vimentin IFs was more pronounced (Fig. 7F). At the end of 8 h, the vimentin IFs were totally collapsed and clustered around the nucleus (Fig. 7I). There was a corresponding change in the other two cytoskeletal filaments, as shown in Figure 7 (bottom row). These results demonstrate a concomitant morphological change as well as a drastic reorganization of vimentin IFs with taxol-induced phosphorylation.

DISCUSSION

Taxol stabilizes MTs and is thus toxic to most mammalian cells. Since the cytoskeletal systems (i.e., the MTs, IFs, and AFs) are interconnected, it is conceivable that MT poisons would disturb the organization of other filamentous networks. It has been extensively reported that MT destabilizing agents, such as colcemids, vinca alkaloids (i.e., vincristine and vinblastine), as well as nocodazole, induce collapse of IF network, however, the effect of taxol, a MT stabilizing drug, has not been investigated. We herein address this question. We show that (1) vimentin is preferentially phosphorylated in taxol-treated 9L RBT cells, (2) this process may be mediated by PKC, and (3) the phosphorylation is accompanied by a massive reorganization of the vimentin IFs. This is a first study demonstrating concomitant hyperphosphorylation and reorganization of vimentin in mammalian cells in response to taxol.

A number of cellular processes are disturbed by taxol and the effects may or may not be MT dependent. Treatment with taxol may lead to activation/inactivation of specific protein kinases. For instance, it has been shown that taxol leads to activation of Raf-1 kinase [Blagosklonny et al., 1997] and also to significant

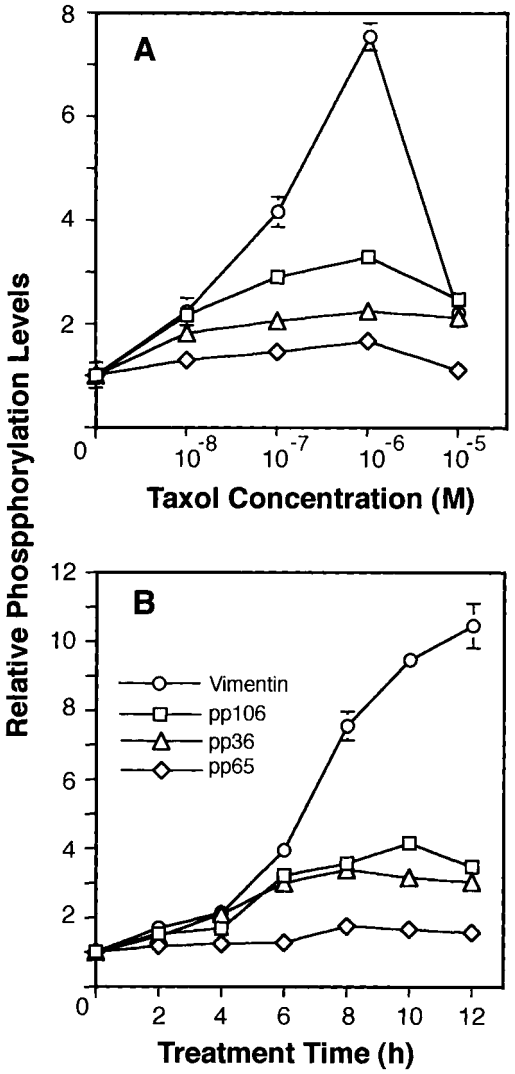

Fig. 4. Dose dependent effects of taxol on phosphorylation of vimentin, pp106, pp65, and pp36 in taxol-treated 9L RBT cells. Autoradiographs as shown in Figures 2 and 3 were scanned and the phosphorylation levels of phosphovimentin, pp106, pp65, and pp36 were presented as the sums of the pixel values after background subtraction and relative to that in the untreated controls. The data are means ±SD of three independent experiments. **A:** Concentration–dependent changes. **B:** Time-dependent changes.

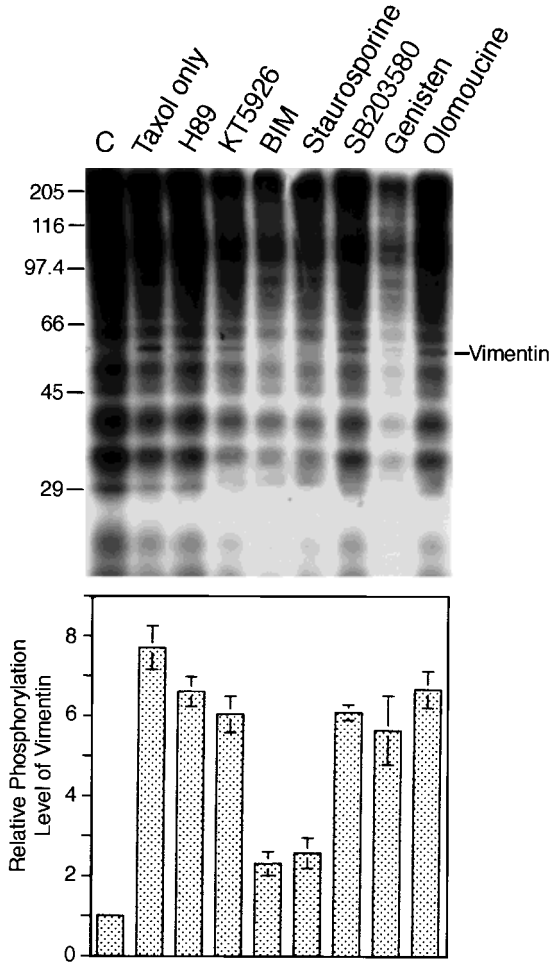

Fig. 5. Effects of protein kinase inhibitors on vimentin phosphorylation in taxol-treated 9L RBT cells. Cells were pre-incubated with the indicated protein kinase inhibitors at concentrations as specified under in Materials and Methods for 6 h. Subsequently, the cells were washed and then treated with taxol, metabolically labeled with [^{32}P]orthophosphate, and processed for gel electrophoresis as for previous experiments. Shown are autoradiograph **(top)** and quantitative data obtained by densitometric scanning **(bottom).**

increase in PKC activity in human lung adenocarcinoma cells [Das and White, 1997]. The drug also activates MAPK in mouse peritoneal macrophages [Ding et al., 1996] but inhibits MAPK and p34cdc2 in human lung cancer cells [Nishio et al., 1995]. Other proteins, such as Raf-1 and Raf-1 kinase, are also activated by taxol and the process coincides with phosphorylation and inactivation of Bcl-2 [Blagosklonny et al., 1995; 1996]. Furthermore, taxol increases nitric oxide synthesis [Jun et al., 1995], mimics the effects of lipopolysaccharide which leads to the induction of a panel of immediate–early genes [Henricson et al., 1995], increases protein tyrosine phosphorylation [Henricson et al., 1995; Wolfson et al., 1997] as well as activation of NF-kappa B [Das and White, 1997]. It is important to note that alteration of protein phosphorylation is implicated in all these processes. Therefore, it is apparent that taxol, directly or indirectly, affects protein kinases/phosphatases in the treated cells. Employing an array of protein kinase inhibitors in conjunction with the taxol treatment, we show that taxol-induced phosphorylation of vimentin is

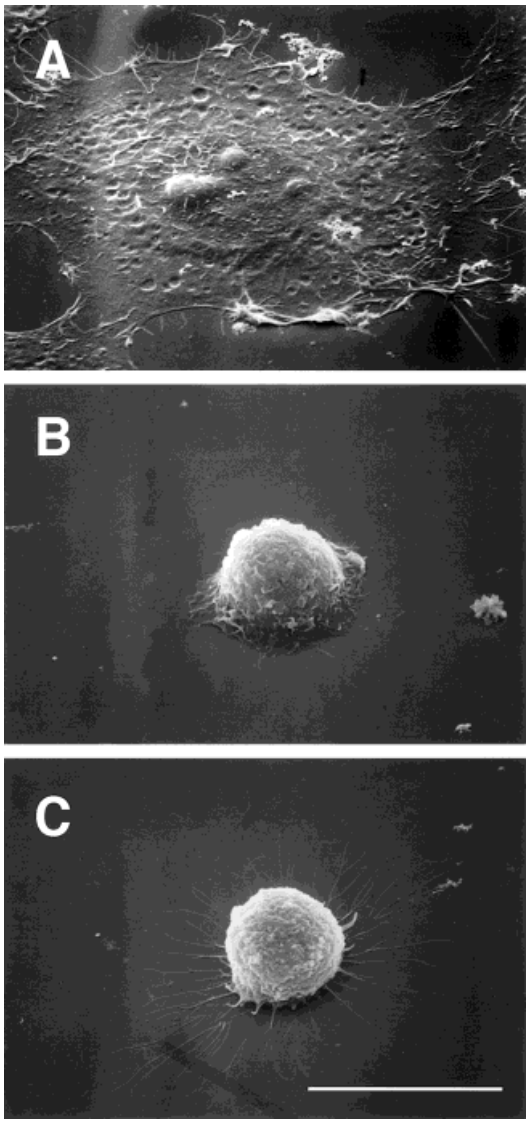

Fig. 6. Morphological changes in 9L RBT cells after taxol treatment. Cells were incubated with 10^{-6} M of taxol for up to 8 h. At 4-h intervals, the cells were fixed, processed for scanning electron microscopy, and viewed by a Hitachi S-2300 SEM at 15 kV. **A:** Untreated control cells. **B:** At 4 h of taxol treatment. **C:** At 8 h of taxol. Bar = 20 μm.

abolished by stauroporine, a general protein kinase inhibitor, as well as by BIM, a PKC-specific inhibitor. This data strongly indicate that phosphorylation of vimentin response to taxol is mediated by PKC. Involvement of PKC in taxol-mediated effects is not unprecedented. Taxol has been shown to increase nitric oxide synthesis in interferon-γ-primed macrophages and this effect is blocked by staurosporine and polymyxin B, both PKC inhibitors [Jun et al., 1995]. Moreover, Das and White [1997] have suggested that PKC is involved in the taxol-induced activation of NF-κB and the activation is blocked by specific PKC inhibitors, BIM and calphostin [Das and White, 1997]. Phosphorylation and the subsequent reorganization of vimentin IF in taxol-treated cells may be partially due to a passive reaction. Based on the reactivity of vimentin toward multiple protein kinases, the presence of multiple phosphorylation sites on vimentin, and the reversibility of the phosphorylation process, we have proposed that vimentin (and perhaps other IF components) may act as a "phosphate sink" during a perturbation of protein phosphorylation–dephosphorylation equilibrium or changes in substrate susceptibility in the stimulated cells [Lai et al., 1994]. Therefore, it is also possible that the phosphorylation and reorganization of vimentin IF induced by taxol is merely due to the conformational changes in vimentin resulting from the disturbance of MT polymerization–depolymerization by taxol. Nonetheless, our data demonstrate a role of PKC in this taxol-induced phosphorylation of vimentin. In addition, we show that the phosphorylation level of two alkaline-stable phosphoproteins, which are likely due to phosphorylation of tyrosine residues [Cooper et al., 1983], is enhanced in taxol-treated cells. Thus taxol may also affect the activity of certain protein tyrosine kinase(s).

Reorganization of the cytoskeletal systems leads to changes in cell morphology. We found that treatment with taxol results in the collapse of IF network, leading to increased cell contractility. Cell shape is maintained by the cytoskeletal networks, and both MTs and IFs are suggested to provide the necessary mechanical strength [Bershadsky and Vasiliev, 1988; Goldman et al., 1996]. The destabilization of IFs by MT poisons is not surprising since the two cytoskeletal systems are closely associated [Goldman et al., 1980; Yang et al., 1992] and may involve IF-associated proteins [Yang et al., 1992; Svitkina et al., 1996], as well as MT-associated and motor proteins [Bloom and Vallee, 1983; Gyoeva and Gelfand, 1991]. In fact, as mentioned previously, all MT destabilizing agents which disassemble MTs also induce the reorganization of vimentin and other type III IFs [see Goldman, 1971; Yang et al., 1992]. This is the first report demonstrating that taxol, a MT stabilizing agent, also disturbs the normal organization of vimentin IFs. This observation further supports the notion that these two cytoskeletal networks are intimately related.

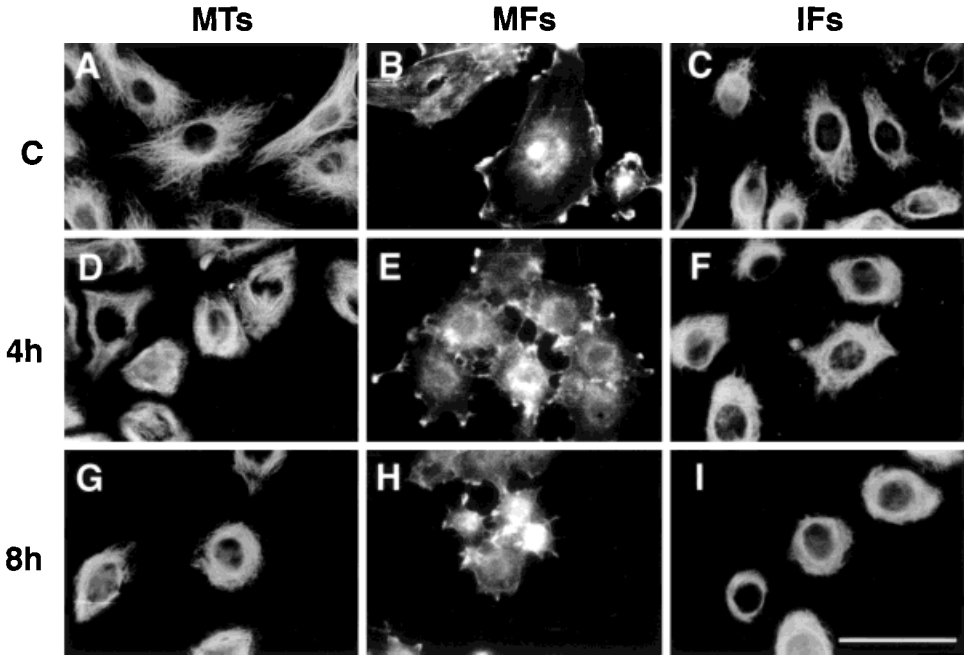

Fig. 7. Distribution of actin, tubulin and vimentin in taxol-treated 9L cells. Cells were incubated with 10^{-6} M taxol for up to 8 h. At 4-h intervals, microtubules (left column) and vimentin IFs (right column) were visualized by indirect immunofluorescent staining with β-tubulin and vimentin antibodies and rhodamine-conjugated goat antimouse IgG. Actin microfilaments were stained directly by rhodamine-conjugated phalloidin (center column). Duration of treatment is indicated at left. Bar = 20 μm.

However, the specific sequential events that lead to the apparent activation of PKC in response to taxol remain unknown and warrant further investigation.

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