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Increased levels of tyrosinated α-, β_{III}-, and β_{IV}-tubulin isotypes in paclitaxel-resistant MCF-7 breast cancer cells☆

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Abstract

Paclitaxel (PTX), the diterpene alkaloid, is a potent anti-cancer drug and is routinely used for the treatment of breast and ovarian cancers. The cellular targets of PTX are microtubules, which are composed of α- and β-tubulin. Development of PTX resistance in patients has been a major problem associated with cancer chemotherapy. In an effort to get insight into this phenomenon of drug resistance, a PTX-resistant cell line from MCF-7 breast cancer cells has been generated. Western analysis of the cell extracts revealed that the resistant cells contain 2-fold higher amount of tyrosinated α-tubulin than those of the wild-type MCF-7 cells. Similar analyses of β-tubulin with the isotype-specific monoclonal antibodies demonstrated that the PTX-resistant cells contain 2.5-fold higher amounts of β_{III} and 1.5-fold higher amount of β_{IV}-tubulin, while no difference was observed in the level of β_I isotype. These results demonstrate for the first time that PTX resistance is associated with an increase in the level of tyrosinated α-tubulin.

Keywords: Tubulin isotypes; Paclitaxel; Tyrosination; Drug resistance; Breast cancer; MCF-7

Tubulin, the structural protein of microtubules, is a heterodimer of two similar polypeptides known as α- and β-tubulin [1–4]. Both α- and β-tubulin exist as multiple isotypes, which exhibit tissue-specific expression (for reviews, see [5–9]). In mammalian system, there are about six α-tubulins designated as α1, α2, α3/7, α4, α6, and α8 and eight β-tubulin isotypes designated as β_I, β_{II}, β_{III}, β_{IVa}, β_{IVb}, β_V, β_{VI}, and β_{VII} [5–9]. Both α- and β-tubulin undergo a number of post-translational modifications that include tyrosination–detyrosination and acetylation of α-tubulin, phosphorylation of β_{III}-tubulin, and polyglutamylation and polyglycylation of both α- and β-tubulin (reviewed extensively in [8]).

Paclitaxel (PTX, also known as taxol), the diterpene alkaloid from the pacific yew tree, *Taxus brevifolia*, is a potent anti-tumor drug [10], which is routinely used for the treatment of a wide range of tumors including the drug-refractile breast and the ovarian tumors [11]. The drug binds to tubulin, stabilizes microtubules [12], and interferes with the microtubule dynamics in vivo [13]. In spite of its useful anti-tumor properties, the use of PTX in cancer chemotherapy is often associated with the development of drug resistance in patients. The mechanism of drug resistance has been explained by various investigators on the basis of either the overexpression of a multidrug transporter P-glycoprotein [14,15], an alteration in the expression of tubulin or specific tubulin isotypes [16–21] or specific mutations in tubulin [22,23]. PTX-resistant ovaian cancer cell lines have been found to overexpress β_I, β_{II}, and β_{IVa} isotypes [23], while the drug-resistant prostate cancer cells overexpress β_{III} and β_{IV} [19,20]. On the other hand, PTX-resistant ovarian cancer cells selected in the presence of a PGP antagonist verapamil were found to contain mutations in β-tubulin without any alterations in the expression of specific tubulin isotypes [22].

Thus, all the published reports indicate that the development of PTX resistance is associated with alterations either in the primary structure or in the level of a specific β-tubulin isotype. Since tubulin is functional

☆ *Abbreviations:* PTX, paclitaxel; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription and polymerase chain reaction.

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only in its dimeric form, it was interesting to study whether the PTX resistance is associated with any alteration in α-tubulin. In an effort to test this, PTX-resistant breast cancer cells were selected in the presence of verapamil to exclude the multidrug-resistant phenotypes. Immunoblot analysis of the cell extract from PTX-resistant cells demonstrates that the level of tyrosinated α-tubulin is elevated significantly as compared to the parental cell line. In addition, the resistant cells were also found to express increased levels of β_{III}- and β_{IV}-tubulin isotypes.

Experimental procedures

Materials. Human breast cancer cells (MCF-7) were obtained from the American Type Culture Collection. All media supplies were purchased from GIBCO BRL. Paclitaxel was a gift from Mathew Suffness, National Cancer Institute, National Institutes of Health. Protease inhibitor cocktail was purchased from Sigma. Monoclonal antibodies to α- and β-tubulin were obtained from Sigma. Rabbit polyclonal antibody to non-tyrosinated tubulin was a gift from Dr. Jeannette Cloe Bulinski and Dr. Gregg G. Gundersen, Columbia University, NY. Horseradish peroxidase (HRP)-labeled secondary antibodies were obtained from Jackson Immunosearch. The chemiluminescent substrate for immunoblot development was obtained from Pierce, Rockford, IL.

Cell growth and preparation of cell extract. Human breast cancer MCF-7 cells were grown in 10% fetal calf serum in DMEM–Ham's F-12 (1:1) mixture in the presence of glutamine and other nutrients in the presence of penicillin–streptomycin–fungisone antibiotics. Cells were washed with warm PBS, trypsinized, and pelleted by centrifugation at 600*g* for 10 min. The cells were washed twice with PBS prior to the preparation of cell extract. The cell pellet was resuspended in RIPA buffer in the presence of 2 mM PMSF along with protease inhibitors and incubated at 27 °C for 10 min prior to centrifugation at 10,000*g* for 30 min.

Preparation of PTX-resistant breast cancer cells. MCF-7 cells were initially grown in the presence of 1 nM of PTX. Verapamil (5 μg/ml) was kept in the selection medium to exclude the multidrug-resistant cells. After a week of growth, the drug concentration was increased to 2 nM and grown for another week. The drug concentration was increased stepwise, each time by a factor of 1.5 times. After 3–4 months of selection, the drug-resistant cells MCF-7^{PTX30} were obtained. After further subcloning, one resistant cell line, MCF-7^{PTX30} 1D11, was obtained as a single colony. This cell line was grown and maintained in complete medium containing 30 nM PTX and 5 μg/ml verapamil. Initial characterization of the cell line will be published elsewhere.

Gel electrophoresis and immunoblotting. Electrophoresis was carried out in 7.5% polyacrylamide gels in the presence of 0.1% SDS according to Laemmli [24]. Protein measurements were done according to Lowry et al. [25]. Samples were boiled for 5 min in Laemmli's sample buffer prior to loading. Immunoblotting was performed as described before [26].

Results

Western analysis

The expressions of different α- and β-tubulin isotypes were studied in cell extracts of breast cancer MCF-7 cell line and the PTX-resistant cell line MCF-7^{PTX30} by SDS–PAGE and immunoblotting with the monoclonal antibodies (see Table 1). The level of α-tubulin was analyzed by immunoblotting using a monoclonal antibody DM1A (Sigma) that recognizes α-tubulin. As shown in Fig. 1, panel A, the level of total α-tubulin was increased significantly. The level of tyrosinated form of α-tubulin was analyzed by immunoblotting using a monoclonal antibody AYN.6D10 which is specific for tyrosinated α-tubulin [26]. As shown in Fig. 1, panel B, the level of tyrosinated α-tubulin is increased 2-fold in the PTX-resistant cells as compared to those of the wild-type MCF-7 cells. On the other hand, the level of detyrosinated tubulin did not change as judged by the immunoblot results with the antibody to detyrosinated tubulin (Fig. 1, panel C).

To study the level of total β-tubulin, the immunoblotting was performed with a monoclonal antibody that recognizes all mammalian β-tubulins (clone 2-28-33, obtained from Sigma). The result shows that the level of total β-tubulin is also increased significantly (Fig. 1, panel D). The levels of individual β-tubulin isotypes were also analyzed by immunoblotting using anti-β_I [27], anti-β_{II} [28], anti-β_{III} [29], and anti-β_{IV} [30] monoclonal antibodies. As shown in the figure, the levels of β_{III}- and β_{IV}-tubulin are increased by 2-fold and 1.5-fold, respectively (panels G and H), while that of β_I is almost unaltered (Fig. 1, panel E). No β_{II}-tubulin was detected either in the wild type or in the PTX-resistant cells by immunoblotting using the anti-β_{II} monoclonal antibody (Fig. 1, panel F).

Discussion

Our results clearly demonstrate that the PTX-resistant breast cancer cells contain significantly higher amounts of tyrosinated α-tubulin than do the wild-type cells. Although the level of total α-tubulin also increased concomitantly, the level of detyrosinated tubulin did not

Table 1
Levels of α- and β-tubulin isotype contents in MCF-7 and PTX-resistant MCF-7^{PTX30} breast cancer cells

Cell line	Relative intensity of the α-tubulin band			Relative intensity of the β-tubulin band			
	Total α-tubulin	Tyrosinated α	Detyrosinated α	β_I	β_{II}	β_{III}	β_{IV}
Wild-type MCF-7	100	100	100	100	ND[a]	100	100
PTX-resistant MCF-7^{PTX30}	155 ± 7[b]	204 ± 2	106 ± 3	100 ± 3	ND	222 ± 7	152 ± 2

[a] Not detected.
[b] Standard deviations are calculated from three independent measurements.

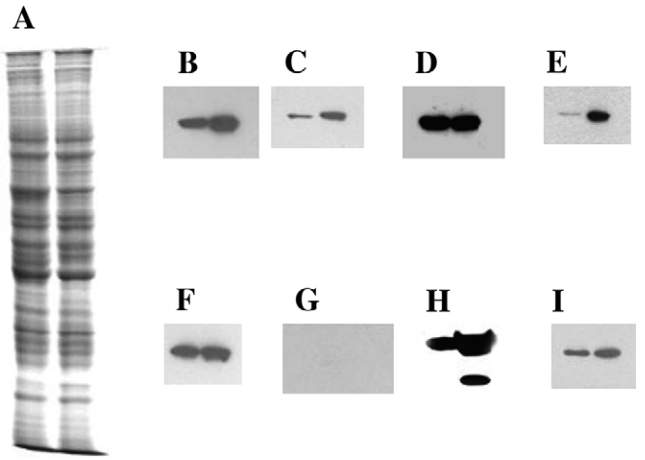

Fig. 1. Western analysis of the cell extracts of PTX-resistant cells with antibodies to α- and β-tubulin isotypes. The cell extracts of wild-type MCF-7 (left lane in each panel) and the PTX-resistant MCF-7^{PTX30} (right lane in each panel) were prepared in RIPA buffer in the presence of protease inhibitors and 2 mM PMSF. The extracts were boiled in Laemmli's sample buffer prior to loading on a polyacrylamide gel (7.5%). Panel A shows the gel stained with Coommassie brilliant blue. Panels B–I are the Western blots of identical samples incubated with the following antibodies: panel B, anti-α-tubulin DM1A; panel C, antibody to tyrosinated α-tubulin AYN.6D10; panel D, polyclonal antibody to detyrosinated α-tubulin; panel E, anti-β-tubulin clone; panel F, anti-β_I SAP; panel G, anti-β_{II} JDR.3B8; panel H, anti-β_{III} and panel I, anti-β_{IV} ONS.1A6. All blots except the antibody to detyrosinated tubulin (panel D) were incubated with HRP-conjugated anti-mouse secondary antibody. The blot for detyrosinated tubulin was treated with HRP-anti-rabbit antibody. The blots were incubated with an enhanced chemiluminescent substrate obtained (Pierce) prior to exposure on Kodak X-omat X-ray film. Forty μg cell extract was loaded in each lane.

change. The analysis of β-tubulin isotypes clearly demonstrated significant increases in the levels of β_{III}- and β_{IV}-tubulin. This is the first report of α-tubulin alteration in PTX-resistant breast cancer cells. The β-tubulin data are clearly consistent with the published data of Cabral et al. [16], Horwitz et al. [21], and Ranganathan et al. [19,20]. Horwitz et al. [21] have shown earlier that PTX-resistant ovarian cancer cells overexpress β_I-, β_{II}-, and β_{IVa}-tubulin. Although I do not see any change in the β_I level, the level of β_{IV} is increased significantly. Recent data show that the β_{IV} isotype in MCF-7 cells is β_{IVb}, because no band corresponding to β_{IVa} was found in the RT-PCR amplified total RNA from those cells (data not shown). Our data on β_{III} and β_{IV} isotype are consistent with those of Ranganathan et al. [19,20], who observed overexpression of both β_{III} and β_{IV} isotypes in PTX-resistant prostate cancer cells [19], while only β_{III} was increased in the estramustine-resistant prostate cancer cells [20].

It is not clear at this point why a specific isotype of tubulin is selectively overexpressed. It may be possible that cells try to survive in a drug microenvironment by selectively overexpressing an isotype that has the lowest affinity for the drug. On the other hand, it may be possible that the cells select those isotypes which impose minimal conformational effects in their PTX complexes. Our previous results have demonstrated that tyrosinated α-tubulin has a higher affinity for PTX than that of detyrosinated tubulin [31]. Thus, overexpression of tyrosinated α-tubulin in PTX-resistant cells cannot be explained on the basis of affinity differences. Whether the overexpression of β_{III}- and β_{IVb}-tubulin is linked to their affinity differences is not very clear. Detailed information on their affinity for PTX are not available. Indirect studies have showed that brain tubulin depleted of the $\alpha\beta_{III}$ isotype assembles much better than the unfractionated tubulin [32]. On the other hand, it is known that dynamic behavior of microtubules plays a major role in PTX resistance. Thus, microtubules assembled from the purified isotypes $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ exhibit differential effects on microtubule dynamics [33]. These two isotypes make microtubules which are least sensitive to PTX. Recent studies have demonstrated that the dynamic instability of microtubules is increased significantly in taxol-resistant lung cancer cells [34].

Thus, it seems interesting that the level of certain isotypes of tubulin becomes elevated in drug-resistant cells. The molecular mechanism by which the level of specific tubulin isotypes is increased in drug-resistant cells is still the subject of further investigation. It will be interesting to study whether selective overexpression of an isotype is due to specific transcription factors. In *Drosophila*, binding sites for the transcription factors *Tinman* and *Engrailed* have been found on the isotype-specific tubulin sequences [35–37]. Furthermore, the involvement of oncogenic growth factors such as EGFR and VEGF-receptor in the selective expression of a tubulin isotype has already been documented [38].

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