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Melanoma cell sensitivity to Docetaxel-induced apoptosis is determined by class III β-tubulin levels

Nizar M. Mhaidat[a,1], Rick F. Thorne[b,1], Charles Edo de Bock[c], Xu Dong Zhang[d], Peter Hersey[d,*]

[a] Department of Clinical Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan
[b] Cancer Research Unit at the University of Newcastle, University Drive, Callaghan Newcastle, NSW 2308, Australia
[c] Queensland Institute of Medical Research, Herston, QLD 4006, Australia
[d] Immunology and Oncology Unit, Room 443, David Maddison Clinical Sciences Building, Cnr. King & Watt Streets, Newcastle, NSW 2300, Australia

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Abstract **We have previously shown that Docetaxel-induced variable degrees of apoptosis in melanoma. In this report, we studied the β-tubulin repertoire of melanoma cell lines and show that class III β-tubulin expression correlated with Docetaxel-resistance. Sensitive cells showed low levels of class III β-tubulin with little microtubular incorporation, whereas class III β-tubulin expression was higher in resistant cells and was incorporated into the cytoskeleton. As proof of concept, abrogation of class III by siRNA reverted Docetaxel-resistant cells to a sensitive phenotype, restoring the microtubular polymerisation response and promoting high levels of apoptosis through Bax activation. These results suggest that phenotypic expression of β-tubulin class III in melanoma may help identify patients with melanoma that can respond to taxanes.**

Keywords: Melanoma; Docetaxel; Class III β-tubulin; Apoptosis; Taxanes

1. Introduction

Given the pivotal importance of microtubules in many cellular functions, they have been the targets for anticancer drugs such as taxanes and vinca alkaloids. By interfering with the dynamics of microtubule assembly, microtubule-binding agents exert profound effects on cellular processes such as gene expression, cell cycle arrest, and apoptosis [1,2]. Taxanes represent an important class of anticancer agents that have anticancer effects in vitro and in vivo against cancers of lung, ovaries, breast, and leukemia [3]. Recently, we have extended these findings showing that Docetaxel induces caspase-dependent apoptosis of some but not all human melanoma cell lines [4].

Resistance of cancer cells to taxanes has been attributed to various mechanisms including over-expression of (multi-drug resistance phenotype) P-glycoprotein that induces efflux of the drug [5,6]. Other studies have reported that taxanes may inappropriately induce activation of pro-survival signaling pathways such as Ras–Raf–MEK–ERK pathway leading to cancer cell resistance [7–9]. Studies have also shown that alteration in the composition and mutations of β-tubulin isotypes, can lead to resistance to taxanes [10,11]. Expression of mutant tubulin or differential expression of certain tubulin isotypes has been shown to correlate with the resistance profile of taxane-resistant cells [10–12]. In some cases, the induced expression of mutant tubulins [13] and β-tubulin isotypes classes III and V [14,15] were shown to confer resistance to paclitaxel. Purified mutant tubulins also have altered polymerisation characteristics [16] and increased expression of classes I, III, and IVa isotypes was reported in paclitaxel-resistant cells [10].

In the present study, we have explored the relation between tubulin isotypes and sensitivity of melanoma cells to Docetaxel and report that high level of class III β-tubulin can confer resistance to Docetaxel-induced apoptosis. Moreover, a mechanism is suggested by the increased incorporation of class III β-tubulin into the microtubular network in Docetaxel-resistant melanoma cells.

2. Materials and methods

2.1. Cell lines

The panel of human melanoma cell lines used have been described previously [17].

2.2. Antibodies and other reagents

Docetaxel (Taxotere), kindly provided by Aventis Pharma S.A (France), was stored as a 100 mM solution in absolute ethanol at −80 °C and diluted immediately prior to use. The polyclonal anti-Bax antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The 107.3 mouse IgG1 antibody was purchased from PharMingen (San Diego, CA). Monoclonal and polyclonal anti-α-tubulin, classes I, III, and IV β-tubulin and β-actin were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia).

2.3. Apoptosis

Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the PI method was carried out as described elsewhere [18] under conditions established from previous work [4].

2.4. Measurement of tubulin polymerisation

Cytosolic and polymerised fractions of tubulin were separated by differential centrifugation as previously described [15]. Briefly cells were lysed in microtubule-stabilizing buffer (20 mM Tris–HCl (pH 6.8), 0.14 M NaCl, 0.5% NP40, 1 mM $MgCl_2$, 2 mM EGTA, and 10 μl/ml protease inhibitor cocktail (Sigma), with 4 μg/ml Docetaxel). The polymerised fraction was obtained by collecting the supernatant

*Corresponding author. Fax: +61 2 49236184.
E-mail address: Peter.Hersey@newcastle.edu.au (P. Hersey).

[1]These authors made an equal contribution towards the work.

Abbreviation: MMP, mitochondrial membrane potential

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fraction after centrifuging the lysate at 10,000 × *g* for 20 min. The pellet (polymerised tubulin) was solubilised before electrophoresis in SDS–PAGE sample buffer.

2.5. Indirect immunofluorescence and confocal microscopy

Melanoma cells seeded onto glass coverslips were fixed after the indicated treatments before permeation with 0.1% Triton X-100. After blocking with 3% bovine serum albumin, cells were immunostained with primary antibodies before sequential detection with Alexa-488 anti-mouse IgG and or Alexa-594 anti-rabbit IgG (Invitrogen, Australia). In some experiments nuclei were visualised by DAPI staining. After mounting in SlowFade Gold reagent cells were examined using a Zeiss Axioplan 2 epifluorescence microscope or Zeiss Axiovert 100 M fitted with the LSM510 confocal system (Oberkochem, Germany).

2.6. Flow cytometry and mitochondrial membrane potential ($\Delta\Psi m$)

Flow cytometric analysis of permeabilised cells for Bax [19] and mitochondrial membrane using JC-1 staining under established Docetaxel treatment conditions as previously described [4].

2.7. Western blot and protein expression analysis

Western blots were performed as described previously [19]. Relative expression was determined against control proteins (GAPDH or actin) using densitometric analysis.

2.8. Small RNA interference (siRNA)

Transfections were performed for 48–72 h as previously described [4] prior to each assay using either SiConTRol Non-targeting SiRNA pool (D-001206-13-20) or the siGENOME SMARTpool for class III β-tubulin (TUBB3) siRNA (Dharmacon, Lafayette, CO).

2.9. Cell viability assays

Cell viability assays were performed using the MTT method. After siRNA treatment cells were seeded at 500 cells/well in 96 well plates and allowed to adhere overnight before addition of Docetaxel. After a further 72 h 30 μl of 5 mg/ml MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide; Sigma) was added to each well for 2 h. The supernatant was removed, the MTT formazan crystals dissolved in 100 μl of DMSO and the optical density read at 550 nm.

2.10. Statistical analysis

The statistical significance of intergroup differences was determined using Student's *t*-test. *P* values $\leqslant 0.05$ and $\leqslant 0.001$ are indicated by * and **, respectively. Regression analyses were performed using the StatView program.

3. Results

3.1. Docetaxel-resistant melanoma cells have low levels of polymerized tubulin

Previous studies have shown that Docetaxel binds to the β-tubulin subunit of the microtubules resulting in their polymerisation [20]. We studied the Docetaxel-induced polymerisation response of microtubules in the IgR3 and MM200 human melanoma cell lines that have been shown previously to be differentially sensitive to this agent [4]. Lysates were prepared from untreated and Docetaxel treated cells and the samples were fractionated into soluble and polymerized tubulin fractions as described in Section 2. Western blotting analysis showed that under normal growth conditions, MM200 cells have a lower level of polymerized tubulin compared to IgR3 cells (Fig. 1A). Densitometric analysis showed that the percentage of polymerized tubulin before and after treatment with Docetaxel was changed from 47% to 86% in IgR3 ($P \leqslant 0.05$) whereas a slight increase from 22% to 31% was seen in

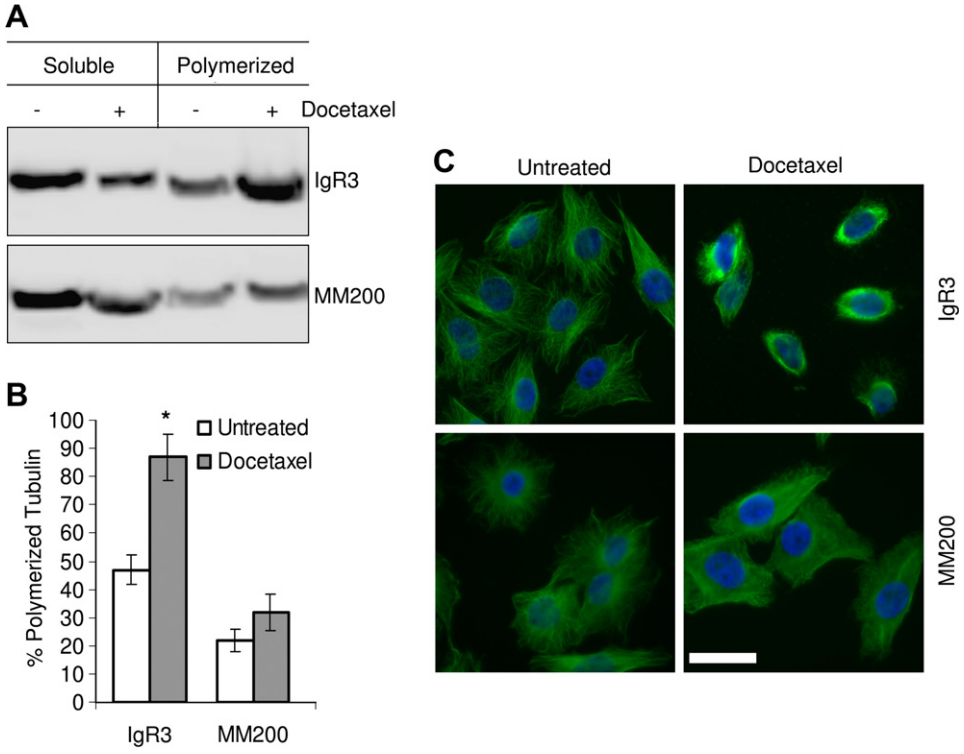

Fig. 1. Docetaxel induces tubulin polymerisation in melanoma cells. (A) Soluble and polymerized fractions of tubulin were separated from cells treated with or without Docetaxel at 20 nM for 3 h and the relative amounts were determined by Western blotting using antibodies against α-tubulin. (B) Data collected from three individual experiments shown in (A) was analysed by densitometry (*Columns*, mean and *bars*, S.E.M.). (C) Cells were also analysed by immunofluorescence microscopy for α-tubulin (green) and nuclear staining (DAPI; blue) under the treatment conditions described for (A). bar = 20 μm.