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Modulation of endogenous β-tubulin isotype expression as a result of human β_{III} cDNA transfection into prostate carcinoma cells

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Summary Increases of individual β tubulin isotypes in antimicrotubule drug resistant cell lines have been reported by several laboratories. We have previously described elevations in β_{III} and β_{IVa} isotypes in estramustine and paclitaxel resistant human prostate carcinoma cells. To investigate further the function of β tubulin isotypes in antimicrotubule drug response, human prostate carcinoma cells that normally have very low to undetectable levels of β_{III} were stably transfected with β_{III} cDNA in pZeoSV system. An 18 bp haemagglutinin (HA) epitope tag was added at the 3′ end prior to cloning into the vector. Cells were transfected with pZeoSV or pZeoSV-β_{III} plasmids and selected in the presence of Zeocin. Immunofluorescent staining of the transfectant cells have shown significant expression and incorporation of HA-tagged β_{III} tubulin into cellular microtubules. Quantitation of Western blots revealed the HA-tagged β_{III} levels to be approximately 7-fold higher than the vector control cells. RT-PCR analysis confirmed the increase at the transcript level and also revealed a collateral increase of β_{II} and β_{IVb} transcripts. Cell viability assays indicated that sensitivity of β_{III} transfected cells to various antimicrotubule agents was similar to vector transfected cells: IC50 values for estramustine, paclitaxel, colchicine and vinblastine were 4 μM, 4 nM, 22 nM and 2 nM, respectively for both cell lines. Thus, overexpression of β_{III} isotype in human prostate carcinoma cells by stable transfection failed to confer antimicrotubule drug resistance to these cells. Counterregulatory increases of endogenous β_{II} and β_{IVb} tubulin isotypes in these β_{III} transfected cells may be a compensatory mechanism used by the cells to overcome the effects of elevated β_{III} levels on the cellular microtubules. These results highlight the difficulty in isolating the contribution of single tubulin isotypes in drug response studies.

Keywords: tubulin; isotype; antimicrotubule agents; drug resistance; transfection

In the past several years many laboratories have studied the significance of α and β tubulin proteins in antimicrotubule drug resistance. These studies have extended to several antimicrotubule agents in various carcinoma cell lines. Early studies showed mutations and alterations in the steady state soluble and polymerized α and β tubulin fractions in drug resistant cell lines (Schibler and Cabral, 1986; Cabral et al, 1986; Minotti et al, 1991). Although the existence of different α and β tubulin isotypes and their tissue specific expression was reported in the past, only recently demonstrated were the effects of individual β tubulin isotypes on overall microtubule functions including assembly, dynamics, drug sensitivity and drug binding. Banerjee et al (1990) have shown that β_{III} isotype depleted tubulin assembles into microtubules at a faster rate than unfractioned tubulin. These microtubules are also more sensitive to paclitaxel-induced assembly compared to unfractioned tubulin (Lu and Luduena, 1993). Subsequent studies revealed alterations in the expression of specific β tubulin isotypes as result of antimicrotubule drug resistance (Haber et al, 1995; Ranganathan et al, 1996; 1998a; 1998b; Kavallaris et al, 1997; Kavallaris et al, 1999). In addition, combination studies have shown that cyclosporin A enhances paclitaxel efficacy in lung carcinoma cell lines by modulating β tubulin isotype composition (Ross and Antoniono, 1999). Recently, Kavallaris et al, (1999) have shown that antisense oligonucleotides to β_{III} isotype sensitized the drug resistant cells to paclitaxel. In addition, mutations in β_{I} isotype were reported in paclitaxel resistant human ovarian carcinoma cells (Giannakakou et al, 1997), breast carcinoma cells (Wiesen and Horwitz, 2000) and non-small-cell lung cancer patients (Monzo et al, 1999) with tumours that were unresponsive to paclitaxel therapy.

Antimicrotubule drug resistance associated changes in tubulin isotypes prompted several investigators to determine the contribution of individual isotypes by transfecting cells and examining their antimicrotubule drug response. Wu et al (1998) have shown that transfection of β_{IVa} isotype into human leukemic cell line failed to confer resistance to paclitaxel. Blade et al (1999) have over-expressed rodent β_{I}, β_{II} or β_{IVb} isotypes in CHO cells and shown that these isotypes do not confer resistance to paclitaxel. Our previous work demonstrated increases in β_{III} and β_{IVa} isotypes in human prostate carcinoma cells that were made resistant to estramustine or paclitaxel (Ranganathan et al, 1996, 1998a). In addition, acute exposures to these agents resulted in elevations of β_{III} levels. To further understand the function of β_{III} isotype in antimicrotubule drug response, parental DU145 human carcinoma cells were transfected with the human β_{III} cDNA. The results presented herein indicate that regulation of tubulin in cells is complex and that attempts to increase levels of a single isotype may lead to compensatory changes in the expression of other isotypes.

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MATERIALS AND METHODS

Construction of expression vectors and selection of transfectants

Human β_{III} cDNA that was previously cloned in our laboratory (Ranganathan et al, 1998b) was inserted into the HindIII and XhoI sites of pZeoSV vector (Invitrogen, Carlsbad, CA). An 18 bp haemagglutinin tag (HA) was added at the C-terminus to distinguish the vector driven β_{III} protein from the endogenous protein. Human prostate carcinoma (DU145) cells were transfected with pZeoSV or pZeoβ_{III} DNA by using superfect (Qiagen, Santa Clarita, CA) according to manufacturer's protocol. Cells were placed in drug selection medium containing 100 µg/mL Zeocin and clones were selected.

Immunofluorescent staining of human β_{III}-transfectant cells

β_{III}-transfectant and vector-transfectant cells were plated onto glass coverslips and allowed to attach. Cells were fixed in 4% paraformaldehyde and stained in the following manner. Cells were treated with 3% BSA in phosphate buffered saline (PBS) for 30 min to block for non-specific binding of the antibodies. Cells were then stained with the following antibodies diluted in 1% BSA/PBS for 90 min at 37˚C: pan α tubulin antibodies, pan β tubulin antibodies (1:200, Sigma Chemical Co., St. Louis, MO), β_{II}, β_{III} and β_{IV} isotype-specific antibodies (1:100, Biogenex, San Ramon, CA) and haemagglutinin antibodies (1:500, BabCo, Richmond, CA). Cells were stained with rhodamine-red-x conjugated antimouse secondary antibodies (1:800, Jacksonville Immunoresearch,) for 45 min at 37˚C, mounted onto slides, and examined. Images were captured with 12 bit cooled CCD (Quantix, Photometrics, Tuscon, AZ) and average pixel intensity values were quantitated and analysed by using Isee software (Inovision Corp., Raleigh/Durham, NC). At least 200 cells were quantited per each antibody stain and the average pixel intensities were calculated.

Western blot analysis of proteins from β_{III}-transfectant and vector-transfectant cells

Cytosolic fractions were prepared from the cells and Western blots were analysed as described previously (Ranganathan et al, 1996). Briefly, cells were lysed for 10 min at 4˚C in PBS containing 1% Triton X100, 0.1% SDS, 0.5% Sodium deoxycholate and protease inhibitors, followed by centrifugation at 10 000 × *g* for 10 min. Protein concentrations of the supernatants were estimated by the Biorad method (Hercules, CA). Proteins were fractionated by 8% polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Bedford, MA). Western blots were probed with antibodies against HA epitope (1:500) and β_{III} tubulin isotype (1:200) and developed using ECL plus chemiluminiscence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Results were quantitated by densitometric scanning of the films.

RT-PCR analysis of β tubulin isotype transcripts from pZeoSV and pZeoβ_{III}-transfectant cells

RNA was isolated from both cell lines using RNAeasy kit from Qiagen Inc (Chadsworth, CA). β tubulin isotype-specific primers for the β_{I}, β_{II}, β_{III}, β_{IVa} and β_{IVb} isotypes and the PCR conditions were described in detail previously (Ranganathan et al, 1996). In addition, vector-specific and HA epitope specific primers were chosen using the primer detective program (Clontech, Palo Alto, CA) and synthesized by the DNA core facility at Fox Chase Cancer Center. PCR products were analysed by agarose gel electrophoresis and quantitated by densitometric scanning.

Cytotoxicity assays

ZeoSV and Zeoβ_{III} transfectant cell lines were plated onto 96-well cell culture plates and allowed to attach. Estramustine was a gift from Kabi Pharmacia, Lund, Sweden (now Pharmacia, Bridgewater, NJ). Paclitaxel, vinblastine and colchicine were purchased from Sigma Chemical Co. Cells were exposed to various concentrations of the drugs for 48 h, fixed and stained with sulforhodamine B, as previously described (Ranganathan et al, 1996). Absorbance was measured at 560 nm and cell survivals in both cell lines were determined.

RESULTS

Expression of β_{III} tubulin isotype by transfection

When the cells were stained with antibodies against HA epitope, β_{III} transfected cells showed significant staining for the HA epitope in the microtubules, indicating β_{III} protein was derived from the pZeoβ_{III} DNA and was incorporated into cellular microtubules (Figure 1D). By contrast, staining for the vector-transfected cells was similar to background level of staining (Figure 1C). When the cells were stained with the antibodies against β_{III} isotype, a 2.4-fold increase in staining was seen in the β_{III} transfectant cells (Figure 1B) compared to the vector transfected cells (Figure 1A). Cells were also stained with antibodies against pan α and pan β tubulin proteins to determine if the over-expression of β_{III} isotype had any affect on the overall β and α tubulin levels (Figure 1 E–H). As shown in the figure, levels of β tubulin levels did not change significantly from vector-transfected cells (Figure 1E) to β_{III} transfected cells (Figure 1F). There was a 1.8-fold increase of overall α tubulin levels in β_{III} transfected cells (Figure 1H) compared to vector transfectant cells (Figure 1G). Quantiation of immunofluorescent staining was done as described in the Methods section and the results are shown in Table 1.

Western blot analysis of the proteins from the transfectant cell lines by use of HA antibodies has confirmed the increase seen by immunofluorescence (Figure 2). Quantitation of the Western blot by NIH image analysis software revealed a 7-fold increase in the HA signal. There were no differences in the overall β tubulin and β tubulin levels between the vector transfected and β_{III} transfected cell lines.

Table 1 Quantitation of immunofluorescent images of β_{III} transfected and vector transfected cells stained with antibodies. Cells were stained, images were captured and average pixel intensities were quantitated as described in the Methods section

Antibody used	Vector-transfectants	β_{III} transfectants
β_{III} tubulin	339 ± 24	828 ± 33
HA-tag	89 ± 17	407 ± 55
Pan β-tubulin	637 ± 67	866 ± 33
Pan α-tubulin	610 ± 42	1120 ± 27

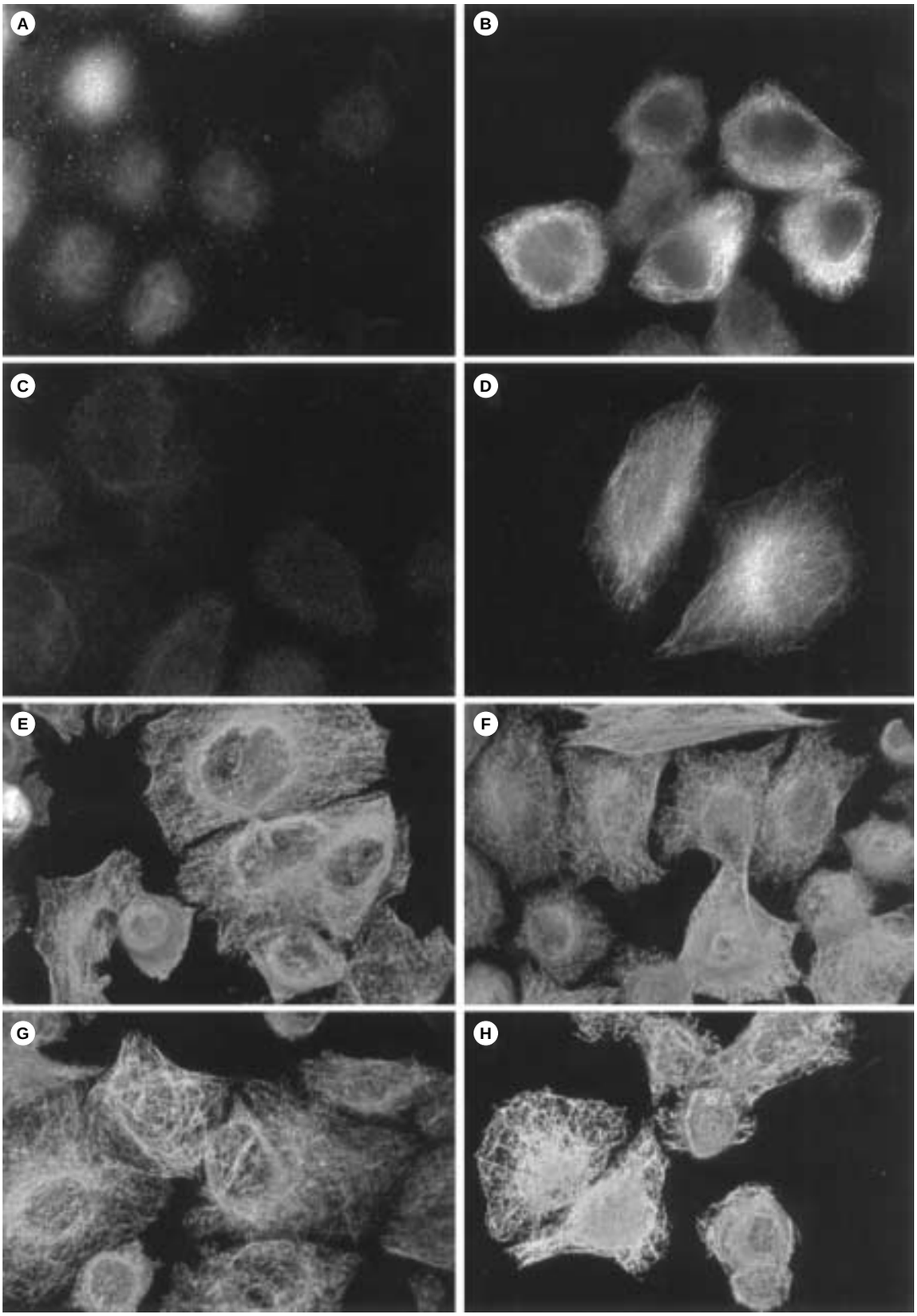

Figure 1 Immunofluorescent staining of pZeoSV (**A, C, E, G**) and pZeoβ_{III} (**B, D, F, H**) transfectants. Cells were plated onto coverslips, fixed and stained with the indicated antibodies as described in the Methods section. (**A, B**) β_{III} tubulin isotype, (**C, D**) HA-epitope tag, (**E, F**) pan β tubulin, and (**G, H**) pan α tubulin

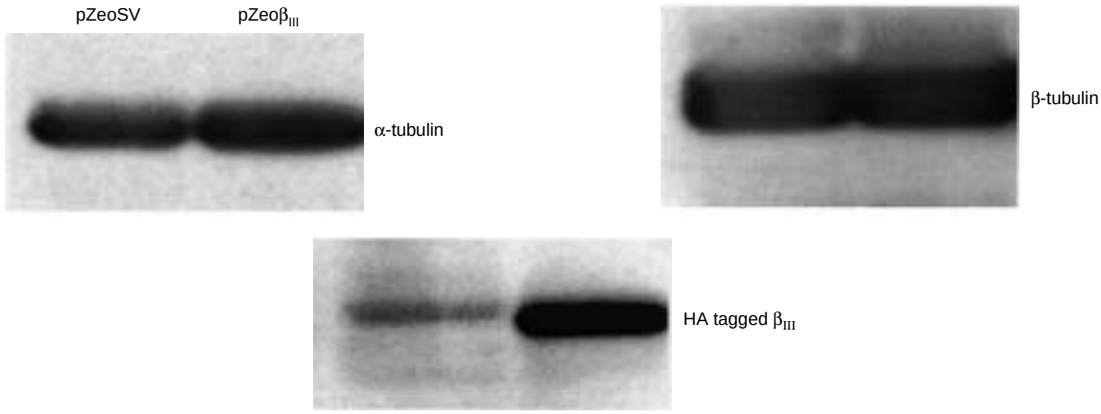

Figure 2 Western blot analysis of pZeoSV and pZeoβ_{III} transfectants. Cell lysates were prepared, run on 8% polyacrylamide gels, transferred onto membranes and probed with the appropriate antibodies as described in the Methods section

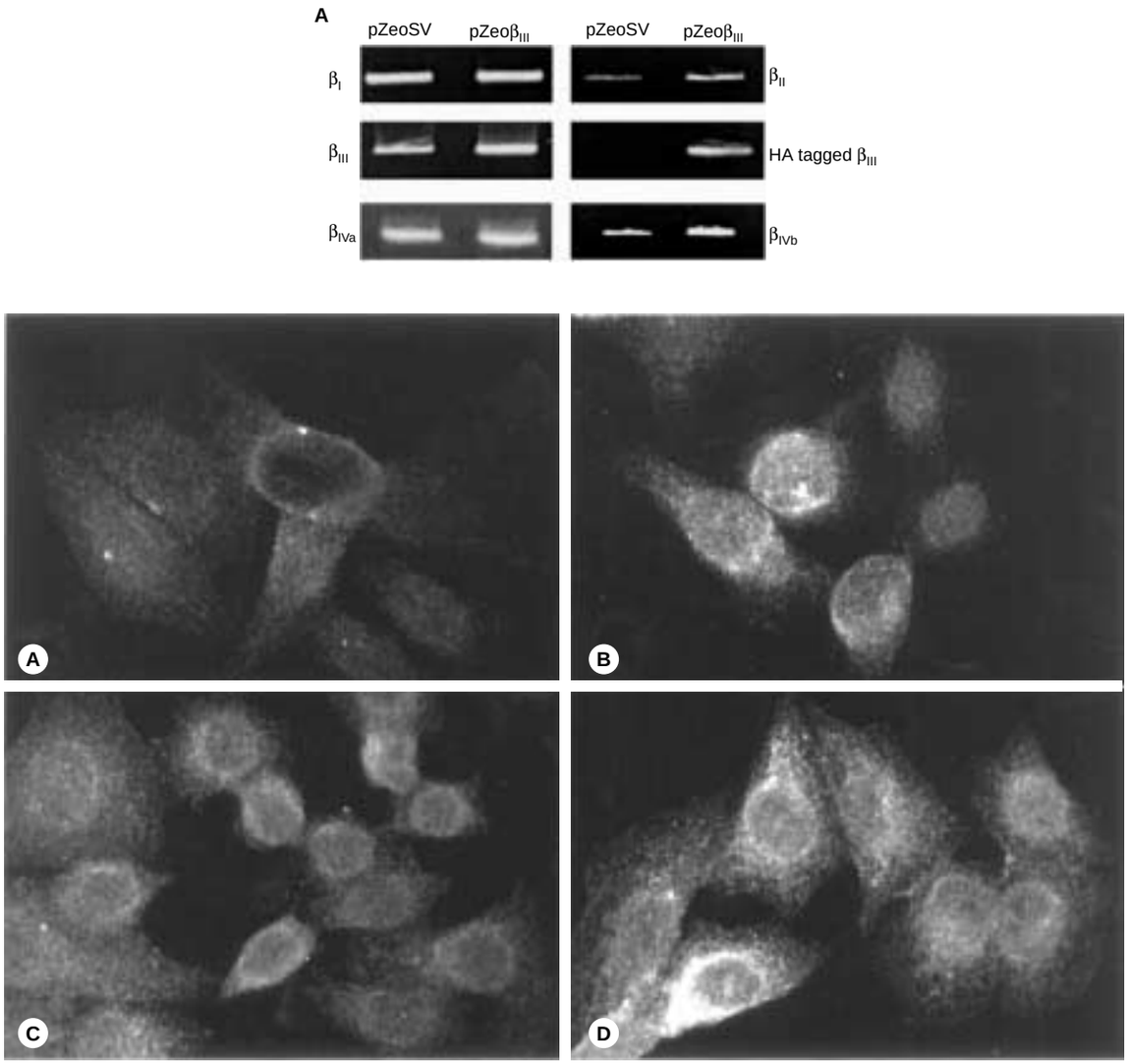

Figure 3 **(A)** RT-PCR analysis of β tubulin transcripts from vector transfected and β_{III} transfected cells. RNA was isolated from both cell lines, RT-PCR was performed with primers for individual β tubulin isotypes and analysed by agarose gel electrophoresis as described in the Methods. **(B)** Immunofluorescent staining of pZeoSV and pZeoβ_{III} transfectant cells. Cells were plated onto coverslips, fixed and stained with antibodies against β_{II} (**A, B**) and β_{IV} (**C, D**) isotypes as described in Methods

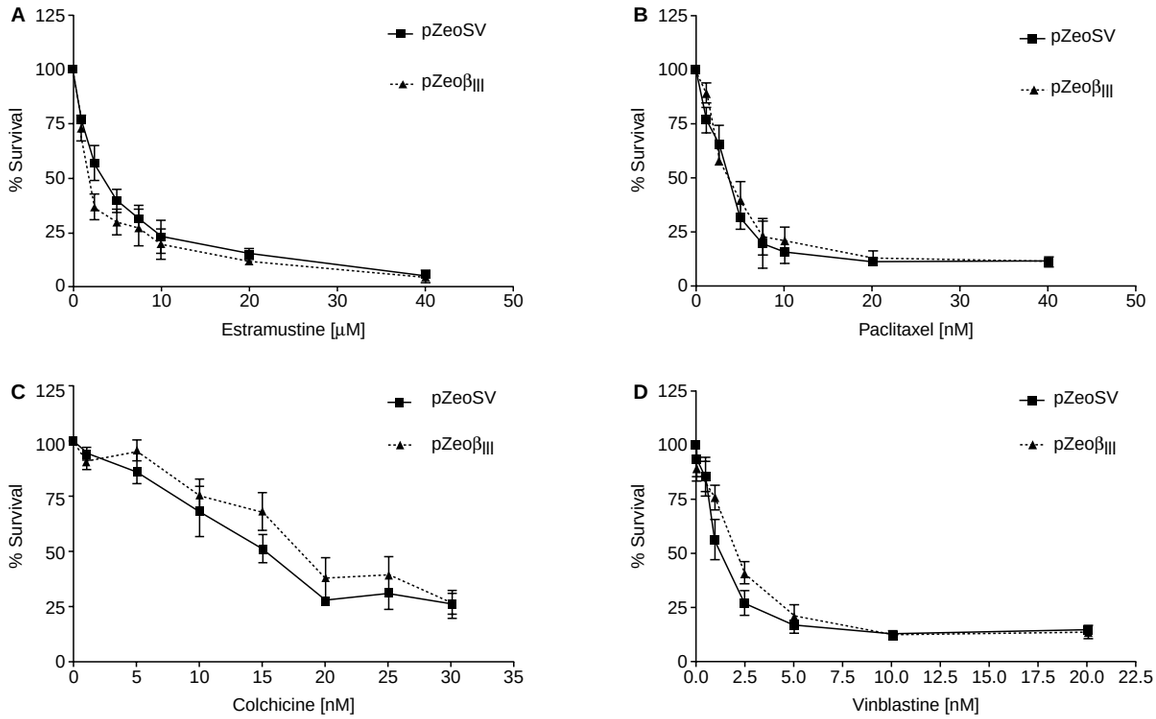

Figure 4 Dose response curves of pZeoSV and pZeoβ_{III} cell lines. Cells were plated onto 96-well plates, allowed to attach and treated with indicated doses of antimicrotubule agents for 48 h. Cells were then fixed and stained with sulforhodamine B and cell survivals were determined as described in the methods. (**A**) estramustine, (**B**) paclitaxel, (**C**) colchicine, (**D**) vinblastine

Coordinated increase of β_{II} and β_{IV} isotype transcripts in β_{III} transfectant cells

RT-PCR analysis of RNA from the transfectant cells has shown that there was a significant increase of β_{III} isotype mRNA, as seen by the elevation of overall β_{III} levels and HA tagged β_{III} levels. (Figure 3A). Quantitation of the image revealed a 3-fold increase of β_{III} transcript levels compared to vector transfectants. There was a significant level of HA tagged β_{III} transcript compared to no signal in the vector transfectants. β_I and β_{IVa} levels were similar in both cell lines. Interestingly, a collateral increase in the levels of β_{II} and β_{IVb} transcripts was seen in the β_{III} transfected cells. The increases were quantified to be 7-fold and 2-fold for β_{II} and β_{IVb} transcripts, respectively. Immunofluorescent staining of the cells with corresponding antibodies has confirmed the increase at the protein level (Figure 3B).

Antimicrotubule drug response of transfectant cells

β_{III} transfectant and vector control cell lines were treated with estramustine, paclitaxel, colchicine and vinblastine for 48 h, and dose response curves were determined (Figure 4). Cytotoxicity profiles of both cell lines were similar for each of the antimicrotubule agents tested, with IC50 values of 4 μM, 4nM and 2nM for estramustine, paclitaxel and vinblastine, respectively. For colchicine, the IC50 values were 15 and 17nM for β_{III} transfected and vector transfected cells. Cell doubling times for β_{III} transfectant and vector control cell lines were found to be very similar, ~22 h indicating that transfection of β_{III} into cells did not alter cell cycle times.

DISCUSSION

Several in vitro studies have demonstrated that the β tubulin isotype composition of microtubules determines the differences in their functions including dynamics, assembly properties and sensitivity to antimicrotubule agents (Banerjee et al, 1990, 1992; Benerjee and Luduena, 1992; Lu and Luduena, 1993, 1994; Panda et al, 1994, 1997; Derry et al, 1997). Because antimicrotubule agents are widely used in cancer therapy, tubulin isotype composition of tumour cells may be an important factor in the success of therapy. Estramustine, vinblastine and paclitaxel have been shown to suppress microtubule dynamics at very low drug concentrations (Jordan et al, 1993, 1996; Dhamodharan et al, 1995; Panda et al, 1997). Work by Lu and Luduena (1993) demonstrated that microtubules lacking the β_{III} isotype polymerized at a faster rate in the presence of paclitaxel than the microtubules from unfractioned tubulin. Derry et al (1997) have shown that suppression of microtubule dynamics by paclitaxel varies according to β tubulin isotype content, with $\alpha\beta_{III}$ microtubules and $\alpha\beta_{IV}$ microtubules being approximately 7-fold less sensitive to the drug than the microtubules from unfractionated tubulin.

Work from many laboratories including our own has shown that cancer cell lines made resistant to antimicrotubule agents have altered tubulin isotype composition. In addition, Kavallaris et al (1999) have shown that antisense oligonucleotides to β_{III} isotype sensitized paclitaxel resistant lung cancer cells to the drug. However, the data presented in this paper demonstrate that transfection and 3-fold over-expression of β_{III} isotype in cells does not confer resistance to estramustine or paclitaxel. Increases of β tubulin isotypes seen in drug resistant cell lines might be due to the

selection with antimicrotubule agents. Another reason for the lack of antimicrotubule drug resistance of β_{III} transfectant cells might be the collateral increases of β_{II} and β_{IVb} levels. Cellular β tubulin levels are under autoregulation through cotranslational degradation of mRNAs (Theodorakis and Cleveland, 1992; Bachurski et al, 1994). In addition, Gonzalez-Garay and Cabral (1995) have shown that over-expression of β_I tubulin in Chinese hamster ovary cells resulted in coordinate increase of α tubulin levels. Based on the collateral increases of β_{II} and β_{IVb} levels in our β_{III} transfectant cells, it is possible to conceive that cells compensate for over-expression of β_{III} by autoregulation of these isotypes.

Tubulin isotypes differ significantly from each other in their assembly properties (Lu and Luduena, 1994). Microtubules from $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ assembled much faster than unfractionated tubulin. $\alpha\beta_{III}$ dimers assembled at a very slow rate and had the highest critical concentration. However, in vitro analysis by Panda et al (1994) has shown that microtubules assembled from $\alpha\beta_{III}$ dimers were more dynamic than the microtubules from $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ isotypes. They further demonstrated that addition of $\alpha\beta_{II}$ dimers to $\alpha\beta_{III}$ dimers suppressed microtubule dynamics. In addition, colchicine binding characteristics of $\alpha\beta_{III}$ dimers were significantly different from $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ dimers, with apparant on-rate constants of 132 ± 5, 30 ± 2 and 236 ± 7 $M^{-1}s^{-1}$ for $\alpha\beta_{II}$, $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ dimers, respectively (Banerjee and Luduena, 1992). The data presented in this paper together with existing in vitro data support the hypothesis that cells might modulate their microtubule functions by altering tubulin isotype composition. The lack of antimicrotubule resistance seen in our β_{III} transfectants, β_{IVa} transfectants (Wu et al, 1999) and the rodent β_I, β_{II} or β_{IVb} transfectants (Blade et al, 1999) altogether point to the conclusion that the β tubulin regulation is highly complex. When cells are transfected with an individual β tubulin isotype, cellular compensatory mechanisms come into play, including altered expression of endogenous β tubulin isotypes, which may alter the effects of the transfected isotype.

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