Docetaxel maintains its cytotoxic activity under hypoxic conditions in prostate cancer cells

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Abstract

Objective: The efficacy of docetaxel has recently been shown to be increased under hypoxic conditions through the down-regulation of hypoxia-inducible-factor 1α (HIF1A). Overexpression of the hypoxia-responsive gene class III β-tubulin (TUBB3) has been associated with docetaxel resistance in a number of cancer models. We propose that administration of docetaxel to prostate patients has the potential to reduce the hypoxic response through HIF1A down-regulation and that TUBB3 down-regulation participates in sensitivity to docetaxel.

Methods: The cytotoxic effect of docetaxel was determined in both 22Rv1 and DU145 prostate cancer cell lines and correlated with HIF1A expression levels under aerobic and hypoxic conditions. Hypoxia-induced chemoresistance was investigated in a pair of isogenic docetaxel-resistant PC3 cell lines. Basal and hypoxia-induced TUBB3 gene expression levels were determined and correlated with methylation status at the HIF1A binding site.

Results: Prostate cancer cells were sensitive to docetaxel under both aerobic and hypoxic conditions. Hypoxic cytotoxicity of docetaxel was consistent with a reduction in detected HIF1A levels. Sensitivity correlated with reduced basal and hypoxia-induced HIF1A and TUBB3 expression levels. The TUBB3 HIF1A binding site was hypermethylated in prostate cell lines and tumor specimens, which may exclude transcription factor binding and induction of TUBB3 expression. However, acquired docetaxel resistance was not associated with TUBB3 overexpression.

Conclusion: These data suggest that the hypoxic nature of a tumor may have relevance as regard to their response to docetaxel. Further investigation into the nature of this relationship may allow identification of novel targets to improve tumor control in prostate cancer patients. © 2010 Elsevier Inc. All rights reserved.

Keywords: Hypoxia; Prostate cancer; Docetaxel; TUBB3; HIF1A

1. Introduction

Hypoxia is progressively emerging as a common feature of prostate tumors. Evidence of tumor hypoxia in the prostate gland has been documented through detection of molecular markers of hypoxia by immunomolecular imaging and physical measurements [1–4]. Tumor hypoxia is progressively associated with reduced oxidative defense, genomic instability, apoptosis resistance, and may be associated with the transition to androgen-independence in prostate cancer [5]. Many conventional anticancer drugs require oxygen for maximal activity [6]. However, changes in cellular phenotype following hypoxic shock may also participate in the reduced cytotoxic properties of anti-cancer agents. While hypoxia activates a variety of cellular messengers, hypoxia-inducible-factor-1 (HIF1A) is the only transcription factor truly regulated by oxygen. It is the binding of this heterodimer (HIF1A, HIF-1β) to hypoxia response elements located in the promoter region of target genes, along with a variety of transcription factors (e.g.,
crease in largely hypermethylated at this site, suggesting that an in-

tivity to docetaxel appears to correlate with results indicate that prostate cancer cells are sensitive to docetaxel and the mechanism of acquired docetaxel resistance. Our potential role of cancer cell lines shock may participate in preferential sensitivity of prostate cancer through hypermethylation of the 3

HIF1A overexpression has been reported to occur in almost 70% of all human tumors including primary and metastatic prostate cancer and their metastases. While HIF1A overexpression has been associated with increased drug-resistance, clinically relevant microtubule-targeting agents (MTA) were recently shown to down-regulate HIF1A protein levels and activity, increasing the sensitivity of tumor cells to these agents. Docetaxel is a MTA currently used for the standard of care first line chemotherapeutic agent for the treatment of hormone refractory prostate cancer. Its effect is however limited by intolerance and the development of taxane-refractory tumors. The mechanism of preferential sensitivity of prostate tumors to docetaxel and this associated acquired docetaxel-resistance remains poorly understood.

Overexpression of Class III β-tubulin (TUBB3) has been associated with taxane resistance in melanoma, pancreatic, ovarian and head and neck cancers. This naturally occurring mutant form of tubulin prevents pro-assembly activity of taxanes on microtubules, thereby reducing their cytotoxic activity. Up-regulation of TUBB3 has been reported in response to hypoxic exposure via HIF1A. Recently it was reported that CpG methylation within the HIF1A response element of the TUBB3 gene blocked transcription factor binding and resulted in down-regulation in gene expression. Hypomethylation of the site was reported in breast and ovarian cancer cell lines, which permitted binding of HIF1A and up-regulation of gene expression. However, paclitaxel resistant cells were largely hypermethylated at this site, suggesting that an increase in TUBB3 expression upon hypoxia is abolished through hypermethylation of the 3' enhancer.

We propose that changes in phenotype following hypoxic shock may participate in preferential sensitivity of prostate cancer cells to docetaxel. We first determined the sensitivity of 2 prostate cancer cell lines in vitro to docetaxel. Activity was correlated with HIF1A gene and protein expression. We next investigated the potential role of TUBB3 down-regulation in sensitivity to docetaxel and the mechanism of acquired docetaxel resistance. Our results indicate that prostate cancer cells are sensitive to docetaxel under both aerobic and hypoxic conditions. This intrinsic sensitivity to docetaxel appears to correlate with HIF1A down-regulation in hypoxic tumors cells and reduced TUBB3 basal expression levels. Finally, we report for the first time evidence of TUBB3 hypermethylation in prostate cancer cell lines and prostate tumor specimens.

2. Materials and methods

2.1. Cell culture and growth conditions

Normal human prostate cell lines PWR-IE and RPWE-1 and human prostate cancer cell lines DU145, 22Rv1, and LnCaP were obtained from the ATCC (Teddington, UK). Frozen stocks were prepared within 2 wk of growth and to ensure authenticity of the lines. Age-matched docetaxel-sensitive (PC3) and docetaxel-resistant (PC3-D12) cell lines were kindly provided by Professor Watson, University College Dublin, Ireland. The lines were maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Globepharm, Guildford, UK) and 1% streptomycin-penicillin (Gibco). Human RC58/T prostate cancer cells were kindly provided by Professor Rhim, Center for Prostate Disease, Bethesda, MD. These cells, along with normal human prostate cell lines PWR-IE and RPWE-1, were routinely maintained in keratinocyte SFM medium (Gibco) supplemented with bovine pituitary extract, recombinant epidermal growth factor, and 1% streptomycin-penicillin (Gibco). DNA was extracted from cell lines using a QIAamp DNA mini kit (Qiagen, Crawley, UK). The lines were grown for a maximum of 10 week and monitored for mycoplasma on a regular basis. The "prostastic" nature of the lines was confirmed by measuring PSA expression (data not shown). Hypoxia (0.5% O2, pO2 < 2 mmHq) was achieved by exposing cells in a 1000 in vivo hypoxic chamber (BioTrace, Bracknell, UK). The cells were exposed to a mixture of nitrogen, CO2 (5%) and compressed air to achieve a 0.5% oxygen concentration. pO2 was monitored with an oxygen probe (OxyLab pO2; Oxford Optronix, Abingdon, UK). Docetaxel, paclitaxel, and vincristine (Sigma-Aldrich, Poole, UK) were dissolved in ethanol to a concentration of 1 mM/mL.

2.2. Cell viability assays

Human prostate cancer cells (2.5 × 10⁴ cells/well) were seeded into 96-well plates prior to a 48 h treatment with increasing docetaxel concentrations (0.1, 1, 10, 100, nM). The sensitivity of the cells was determined using the 96-non radioactive MTT reagent (Promega, UK) according to manufacturer’s instructions. Cell viability in the treated plates was compared with that measured in untreated cells to calculate the surviving fraction.

2.3. Clonogenic assays

Cell survival was evaluated using a standard colony-forming assay. 1,000–10,000 cells/well were plated onto 6-well plates prior to a 48 h chemotherapeutic treatment under aerobic or hypoxic conditions. Two weeks later, the plates were stained (70% ethanol, Cristal violet; Sigma-Aldrich, Poole, UK) and the colonies were counted. The response of aerobic cells was used as a control. The plating efficiency was calculated as the ratio of the number of colonies counted over the number cells. The surviving fraction in the treated wells was subsequently calculated as the ratio of the number of clones counted over the number of cells plated corrected with the appropriate plating efficiency.
2.4. RNA isolation and quantitative RT-PCR analysis

Quantification of HIF1A and TUBB3 mRNA levels was performed in triplicate by a real-time fluorescence detection method as described previously [21]. In brief, after RNA isolation with an RNeasy Mini Kit (Qiagen, Valencia, CA), 2 μg of total RNA was converted to cDNA with a first strand high capacity cDNA reverse transcription kit (Applied Biosystems Ltd., Warrington, Cheshire, UK). The HIF1A and TUBB3 genes and endogenous control gene (PGK1) were -amplified separately using TaqMan real-time PCR (Applied Biosystems). Relative gene expression was determined by applying the arithmetic formula 2-ΔΔ CT.

2.5. Western blot analysis

HIF1A protein expression was determined in whole cell lysates of aerobic and hypoxic 22Rv1 and DU145 cells treated with docetaxel or 5-fluorouracil. The cells were scraped under hypoxic conditions and stored on ice. The pellet was resuspended in lysis buffer [22]. Protein was extracted, subjected to polyacrylamide gel electrophoresis, and transferred to nylon/nitrocellulose membranes. The membranes (Amersham, Little Chalfont, UK) were then probed with anti-HIF1A primary antibody (Cell Signaling Technologies, Hitchin, UK, 1:1000 dilution) and a secondary antibody, polyclonal goat anti-rabbit IgG HRP-linked antibody (Cell Signaling, 1:1000 dilution). The Pierce Luminol kit (Pierce, Northumberland, UK) was used for protein detection. Membranes were stripped prior to reprobing with a mouse monoclonal anti-actin antibody (1:10,000, Sigma-Aldrich, Poole, UK).

2.6. Prostate tissue specimens

CaP tissue specimens (n = 8) from men undergoing radical prostatectomy for primary prostate cancer were obtained through the histopathology archive dating from 1999 to 2006 at the Adelaide and Meath incorporating the National Children’s Hospital, as described previously [23]. Genomic DNA was extracted using a RecoverAll Total Nucleic Acid Isolation Kit (Ambion Inc., Austin, TX) according to manufacturer’s instructions and quantified using a Nanodrop-1000 spectrophotometer (Labtech International, Ringmer, UK).

2.7. TUBB3 methylation

Hypermethylation of the putative HIF1α response element located within a 3’ enhancer region of the TUBB3 gene was analyzed by pyrosequencing. Genomic DNA isolated from tissue specimens (50 ng) and cell lines (500 ng; by use of a QIAamp DNA blood minikit (Qiagen)) was bisulfite modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) and eluted into 50 μl 1 X TE buffer. The CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA) was employed as a positive methylated control. The EpiTect unmethylated DNA (Qiagen, UK) was used as an unmethylated DNA control. Bisulfite-treated DNA was PCR amplified for 45 cycles with a biotinylated primer using the PyroMark PCR kit (Qiagen, UK) in a final volume of 25 μl. Forward primer: 5’-BIOTIN-agggtttttttGCGttttttgtat-3’; reverse primer: 5’-aattacctctaaaatataaacaacaaacatt-3’. The PCR product (20 μl) was immobilized on streptavidin sepharose beads (GE Healthcare, Little Chalfont, UK), washed, and denatured using the pyrosequencing vacuum prep tool (Qiagen, UK), according to the manufacturer’s guidelines. Then, 0.3 μM pyrosequencing primer (5’tgtagtttgtggt-3’) was annealed to the purified single-stranded PCR product and pyrosequencing was performed using the PyroMark Q24 system (Qiagen, UK). The degree of methylation was calculated using the PSQ HS 96A 1.2 software, under the CpG mode (Qiagen, UK).

2.8. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was calculated using SPSS software ver. 14.0, (SPSS Inc., Chicago, IL). Differences in surviving fraction and relative gene expression were compared using Student’s t-tests or an analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean.

3. Results

3.1. Sensitivity of prostate cancer cells to docetaxel

We initially generated dose response curves of both 22Rv1 and DU145 cells treated with increasing concentrations of docetaxel. Survival was measured using an MTT assay (Fig. 1A). The sensitivity of DU145 and 22Rv1 cells to docetaxel was similar. We next chose a concentration of 1nM for more specific determination of the sensitivity of each cell line to docetaxel using clonogenic assays (Fig. 1B). DU145 cells were significantly more sensitive to docetaxel than 22Rv1 cells (P = 0.014).

3.2. Docetaxel maintains its activity in hypoxic prostate cancer cells

To determine whether docetaxel preferentially maintained their cytotoxicity under hypoxic conditions, both cell lines were treated with 1nM docetaxel for 48 h under aerobic or hypoxic conditions. The response of cells to treatment with a non-MTA agent, 5-fluorouracil (5-FU, 100 nM) was in addition used as a control to determine whether the response was specific to docetaxel (Fig. 2). Hypoxia-induced chemoresistance was evident in 22Rv1 cells treated with 5-FU only (P = 0.04). Hypoxic 22Rv1 and DU145 cells were as sensitive to docetaxel as aerobic controls.
3.3. Sensitivity to docetaxel in hypoxia correlates with HIF1A down-regulation

We next investigated whether increased sensitivity of prostate cancer to docetaxel under hypoxic conditions correlates with deregulation of HIF1A protein and gene expression. 22Rv1 and DU145 cells were treated with either docetaxel (1 nM) or 5-FU (100 nM) for 48 h under both conditions prior to total protein or mRNA extraction. The response of aerobic untreated cells was used as a control. Hypoxia-selective stabilization of the HIF1A protein was evident in hypoxic cell lysates of both 22Rv1 and DU145 cells (Fig. 3). Docetaxel appeared to down-regulate HIF1A protein levels in both hypoxic cell lines. The 5-FU treatment resulted in undetectable HIF1A levels in 22Rv1 cells but appeared to increase HIF1A stabilization under hypoxic conditions. The HIF1A gene was down-regulated 5- and 16-fold, respectively, in 22Rv1 and DU145 hypoxic untreated cells, compared with aerobic controls. Treatment with docetaxel did not modify the reduction in HIF1A expression in both hypoxic lines. In aerobic controls, HIF1A expression appeared to be up-regulated in response to 5-FU treatment in 22Rv1 cells and down-regulated in DU145 cells (Fig. 4A, B). Time course experiments confirmed down-regulation of HIF1A within 4 h of hypoxic exposure in both cell lines (Fig. 4C, D). At this time point, the reduction in HIF1A expression levels was again not modified in response to both docetaxel and 5-FU treatment in these hypoxic cells (Fig. 5E, F). At this 4 h time point, in aerobic controls, both docetaxel and 5-FU appeared to induce an up-regulation of HIF1A expression in 22Rv1 and down-regulation in DU145 cells.

3.4. Acquired docetaxel resistance is not associated with hypoxia-induced chemoresistance

We next investigated whether transition to docetaxel resistance is associated with increased chemoresistance under hypoxic conditions in an isogenic pair of age-
matched PC3 and docetaxel-refractory PC3 lines (PC3-D12). Both cell lines were treated with docetaxel under aerobic and hypoxic conditions for 48 h. Clonogenic survival was measured and compared with that obtained in cells treated with 2 other MTAs (vincristine and paclitaxel) and to a non-MTA, 5-FU (Fig. 5). Age-matched PC3 cells were significantly more sensitive to docetaxel than vincristine, paclitaxel, and 5-FU under both aerobic (ANOVA, \( P < 0.0022 \)) and hypoxic (ANOVA, \( P < 0.001 \)) conditions. PC3-D12 cells were significantly more sensitive to docetaxel than any of the three other drugs tested under aerobic conditions only (ANOVA \( P = 0.04 \)). Hypoxic treatment was not associated with increased docetaxel resistance in either of these cell lines (age-matched PC3, \( P = 0.44 \); PC3-D12, \( P = 0.08 \)). PC3-D12 were significantly more resistant to docetaxel than PC3 cells under both aerobic (\( P = 0.01 \)) and hypoxic (\( P = 0.001 \)) conditions.

3.5. Sensitivity to docetaxel correlates with down-regulation of \textit{TUBB3} 

To determine whether basal \textit{TUBB3} gene expression correlates with increased intrinsic sensitivity to docetaxel, relative \textit{TUBB3} mRNA levels were measured in aerobic and hypoxic cells (4 h) treated with docetaxel (1 nM). The response of aerobic untreated cells was used as a control. Basal \textit{TUBB3} expression was down-regulated (2.1-fold) in DU145 compared with 22Rv1 (Fig. 6A). \textit{TUBB3} expression was down-regulated (2-fold) in response to hypoxic exposure (4 h) in both docetaxel-treated and untreated cells (Fig. 6B, C). To determine whether this response was docetaxel-specific, \textit{TUBB3} expression was also examined in 22Rv1 and DU145 cells treated with 5-FU (100 nM). Relative \textit{TUBB3} mRNA levels were similar to that of docetaxel-treated cells under both aerobic and hypoxic conditions. To investigate a potential role for \textit{TUBB3} in acquired chemoresistance, \textit{TUBB3} mRNA levels were next mea-
3.6. Down-regulation of TUBB3 in hypoxia correlates with hypermethylation of the putative HIF1A response element

Finally, hypermethylation of a putative HIF1A response element within the 3' UTR of the TUBB3 gene was determined in a panel of normal (PWR1E, RWPE1) and malignant (LNCaP, PC-3, DU145, 22RV1, and RC58) prostate cell lines (Fig. 7). All lines tested displayed evidence of hypermethylation, however, amounts of 5-methylcytosine detected were higher in the cancer (80% in 22RV1, DU145, and PC-3) than in both normal lines (50%). The amount of 5-methylcytosine detected in LNCaP (32.61%) was lower than the other cell lines. Evidence of hypermethylation was also observed in all 8 prostate patient tumor specimens sequenced, although there was variation in the amount of methylation. Two tumors (T5 and T8) displayed levels similar to the LNCaP cell line. Culturing of the cells in acute and chronic hypoxia did not alter the methylation status (results not shown).

4. Discussion

Hypoxia and the stabilization of HIF1A is a known cause of treatment resistance in solid tumors [24]. Our data confirmed that 2 prostate tumor cell lines were sensitive to treatment with docetaxel. This sensitivity was maintained under conditions of hypoxia and correlated with taxane-independent down-regulation of the HIF1A gene in both cell lines during hypoxic exposure. Relative HIF1A mRNA levels were reduced within 4 h of hypoxic exposure and were undetectable at 24 h. This rapid degradation may indicate the importance of the HIF1A gene in the initial phases of the hypoxic response. The mechanism behind this rapid down-regulation was not investigated but may be dependent on oxygen availability.

We previously reported that HIF1A protein expression in 22Rv1 and DU145 cell peaks between 4 and 8 h of hypoxic exposure, and is undetectable following 48 h of exposure [25]. We now show that after 6 h in hypoxia, treatment with docetaxel appears to reduce HIF1A expression in metastatic DU145 cells to a greater extent than in primary 22Rv1 cells.
This may be due to the mitotic fraction of hormone refractory prostate cancer cell lines such as DU145, which has been shown to be less than 2% [10]. Docetaxel has indeed been proposed to exert a greater effect in metastases of prostate cancer through inhibition of HIF1A, rather than anti-mitotic effects in primary tumor systems [10].

Although various authors have described the mechanisms of taxane resistance pathways, there is little evidence regarding clinical studies of taxane resistance in prostate cancer [15]. We sought to investigate whether the transition to docetaxel resistance was associated with increased chemo-resistance in hypoxia. We used a docetaxel resistant metastatic cell line (PC3-D12) and a docetaxel sensitive age matched control (PC3). The PC3-D12 cell line was significantly more resistant to treatment with docetaxel than the control PC3 line under both aerobic and hypoxic conditions. Hypoxia was not associated with increased docetaxel resistance. Age matched control PC3 cells were significantly more sensitive to docetaxel than other MTAs (vincristine and paclitaxel) as well as the non-taxane control 5-FU in both aerobic and hypoxic conditions. The mechanisms of this docetaxel-specific resistance were not investigated. Docetaxel is a synthetic analogue of paclitaxel. The compounds differ in their chemical structure [26] but both compounds exhibit similar mechanisms of action [27]. Docetaxel was demonstrated to be a more potent anti-cancer drug than paclitaxel in a number of cancer models in vitro and in vivo (reviewed in [28,29]); possibly via prolonged intracellular retention [30]. Yet resistance to docetaxel does not necessarily induce resistance to paclitaxel [31]. Further research is required to characterize this phenomenon.

Class III \(\beta\)-tubulin (TUBB3) overexpression was proposed to induce taxane resistance by preventing the ability of the compounds to destabilize microtubules [16–19]. Down-regulation of TUBB3 expression was associated with increased taxane sensitivity [16,21]. TUBB3 expression was 2-fold lower in the aerobic untreated DU145 cell line compared with the aerobic primary untreated 22Rv1 cells. The reduced expression of TUBB3 observed in DU145 cells may contribute to our observation of a greater sensitivity to treatment with docetaxel in metastatic DU145 cells in clonogenic survival assays. We subsequently measured TUBB3 mRNA levels in cells exposed to hypoxia. Hypoxia was indeed proposed as a possible inducer of TUBB3 expression in ovarian and cervical cancer cell lines [20]. Hypoxic exposure down-regulated the gene in both untreated and treated cells and to a greater extent (2-fold) in the primary 22Rv1 cell line. This down-regulation of TUBB3 expression in primary prostate cell lines is consistent with the continued sensitivity of hypoxic prostate cells to docetaxel. TUBB3 over-expression, however, did not appear to play a role in the acquired docetaxel resistance in the PC3-D12 cell line.

It was recently reported that hypoxia-dependent TUBB3 expression is abolished in taxane-resistant cells through methylation of the 3′ enhancer [17]. TUBB3 methylation may represent a potential pretreatment molecular marker for preferential response to docetaxel in patients presenting...
with prostate tumors. CpG methylation within the HIF1A response element blocked transcription factor binding and resulted in down-regulation of expression of target gene TUBB3 [20]. Intriguingly, the response element is not present in the 5′ CpG island, but is located 168 bp into the 3′ UTR of the TUBB3 gene, within a downstream enhancer region. Hypermethylation of this site was evident in a panel of prostate cell lines and 8 tumor specimens. This is the first report of TUBB3 hypermethylation in prostate cancer. These results highlight differential hypermethylation levels between normal (50%) and malignant (80%) cell lines, with particularly low amounts of 5-methylcytosine detected in LNCaP cells (30%). Hypermethylation levels were also variable in tumor specimens. High levels of hypermethylation at this site did not correlate with the reduced TUBB3 expression levels in DU145 compared with 22Rv1 cells, however, the gene was down-regulated in hypoxia in both cell types. These findings would support further evaluation of TUBB3 methylation in a larger cohort of patients.

4. Conclusion

Our data suggest that the combination of the hypermethylation of target genes such as TUBB3 at their HIF1A binding site with the HIF1A targeting property of docetaxel may represent a possible expansion in the administration of docetaxel in the management of prostate cancer.

References


