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Glutathione-S-transferase pi 1(GSTP1) gene silencing in prostate cancer cells is reversed by the histone deacetylase inhibitor depsipeptide

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ABSTRACT

Gene silencing by epigenetic mechanisms is frequent in prostate cancer (PCA). The link between DNA hypermethylation and histone modifications is not completely understood. We chose the *GSTP1* gene which is silenced by hypermethylation to analyze the effect of the histone deacetylase inhibitor depsipeptide on DNA methylation and histone modifications at the *GSTP1* promoter site. Prostate cell lines (PC-3, LNCaP, and BPH-1) were treated with depsipeptide; apoptosis (FACS analysis), *GSTP1* mRNA levels (quantitative real-time PCR), DNA hypermethylation (methylation-specific PCR), and histone modifications (chromatin immunoprecipitation) were studied. Depsipeptide induced apoptosis in PCA cells, but not a cell cycle arrest. Depispeptide reversed DNA hypermethylation and repressive histone modifications (reduction of H3K9me2/3 and H3K27me2/3; increase of H3K18Ac), thereby inducing *GSTP1* mRNA re-expression. Successful therapy requires both, DNA demethylation and activating histone modifications, to induce complete gene expression of epigenetically silenced genes and depsipeptide fulfils both criteria.

1. Introduction

Epigenetic alterations play an important role in carcinogenesis. Aberrant promoter CpG island hypermethylation causes transcriptional gene silencing [1], and has been documented for a number of genes in prostate cancer (PCA) [2]. Histone modifications are also important regulators of transcriptional activity; so far, they have been less comprehensively studied in PCA despite the fact that changes in DNA methylation are closely related to histone alterations and both mechanisms interact in the regulation of gene expression. Posttranslational modifications (e.g. acetylation and methylation) occur on the N-terminal tail of the histone, and function by disrupting chromatin contacts and affecting the recruitment of various proteins to the chromatin, thereby modifying the transcriptional activity. For example, histone H3 lysine 9 methylation (H3K9me) and histone H3 lysine 27 methylation (H3K27me) are linked with transcriptional repression, whereas histone H3 lysine 4 methylation (H3K4me) is connected with transcriptional activation. In addition, histone lysine methylation exists in the form of mono (me1), di (me2) and tri (me3) methylation. Histone lysine acetylation in general leads to gene activation [3].

Epigenetic alterations are reversible. Numerous studies demonstrated re-expression of epigenetically silenced genes after treatment with DNA-methyltransferase (DNMT) inhibitors (e.g. decitabine) and histone deacetylase (HDAC) inhibitors (e.g. vorinostat) in cancer cell lines [4–6]. However, side effects limit the use of these classical inhibitors.

A novel HDAC inhibitor, depsipeptide (romidepsin, FK228), was recently approved for the treatment of cutaneous T-cell-lymphoma and is relatively well tolerated [7]. Depsipeptide induces histone acetylation by inhibiting HDAC class I [8]. The acetylation of the histones alters the chromatin structure and the recruitment of enzymes. Depsipeptide reduces the binding of DNMT1 to the promoter region of tumor suppressor genes and the expression of histone methyltransferases [9]. Thus, depsipeptide may also be useful for epigenetic therapy of PCA.

Epigenetic alterations in PCA are best characterized for the *GSTP1* gene: The CpG island at the promoter of *GSTP1* is unmethylated in the normal prostate epithelium, but methylated in PCA cells [10]. DNA hypermethylation is associated with a repressive histone modification pattern (i.e. H3K9me2) at the *GSTP1* promoter in LNCaP cells [11].

In this study we explore the effect of depsipeptide on the cell cycle in different prostate cell lines. We chose *GSTP1* as a model gene to analyzed how depsipeptide influences DNA methylation, histone modifications and *GSTP1* gene expression.

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2. Materials and methods

2.1. Cell culture and depsipeptide treatment

We investigated LNCaP (hypermethylated/silenced *GSTP1* mRNA), PC-3 (partially methylated/expressed *GSTP1*) and BPH-1 (unmethylated/expressed *GSTP1*) because of its known DNA hypermethylation and gene expression status in PCA cell lines. All cell lines were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cell lines were cultured at 37 °C and 5% CO_2. PC-3 and BPH-1 cells were grown in RPMI 1640 medium, whereas LNCaP cells were maintained in Quantum 263 with L-glutamine (all cell media: PAA Laboratories, Pasching, Austria). Fetal bovine serum (10%) and 0.4% Penicillin–Streptomycin (both PAA Laboratories) were added to all cell cultures. BPH-1 cells were supplemented with 1% insulin transferrin sodium and 0.0048% testosterone (Sigma, St. Louis, MA, USA). Subconfluent cells were treated with 5 nM depsipeptide (Gloucester Pharmaceutics, Cambridge, MA, USA) for 48 h.

2.2. Cell cycle analysis

Cells were grown as reported above in cell culture flasks (175 cm^2) at an initial concentration of 2×10^5. After treatment, cells were treated with trypsin (PAA Laboratories), washed twice with PBS (PAA Laboratories) and fixed in ethanol overnight. After incubation with saponine (Sigma) for 15 min at room temperature, cells were stained with propidium iodide (60 μg/ml) and incubated with RNAse (Sigma) for 30 min at room temperature. Cell cycle analysis was performed on a FACScanto flow cytometer (BD Biosciences, San Jose, USA) using the FACSDiva 5.0.3 software.

2.3. RNA isolation and mRNA quantification

Total RNA was extracted using the RNA PureLink Mirco-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA (1.5 μg) using the SuperScript™ III First-Strand Synthesis Kit (Invitrogen). cDNA (1 μl) was amplified in triplicate using SYBR GreenER qPCR Supermix (Invitrogen) on an ABI PRISM *7900HT* (Applied Biosystems, Foster City, CA, USA). Relative *GSTP1* mRNA expression (primer sequences: forward 5′-TAT-AAG-GCT-CGG-AGG-CCG-3′; reverse 5′-GCG-TAC-TCA-CTG-GTG-GCG-A-′3) was calculated using the delta-delta CT formula and normalized to *HPRT1* (primer sequences: forward 5′-GAC-CTT-GAT-TTA-TTT-TGC-ATA-CC-3′; reverse 5′-CAT-CTC-GAG-CAA-GAC-GTT-CA-′3).

2.4. DNA isolation, bisulphite treatment and methylation-specific PCR

The Pure Link Genomic DNA Mini Kit (Invitrogen) was used to isolate DNA. DNA (500 ng) was subjected to a sodium bisulphite modification using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. *GSTP1* methylation was determined using quantitative methylation-specific PCR on an ABI PRISM *7900HT (Applied Biosystems)*. Bisulphite-modified DNA (1 μl) was amplified using SYBR Green PCR Master Mix (Applied Biosystems). *GSTP1* (primer sequences: forward 5′-GTC-GTG-ATT-TAG-TAT-TGG-GGC-3′; reverse 5′-CTA-ATA-ACG -AAA-ACT-ACG-ACG-ACG-′3) methylation was normalized to the amount of input DNA (*ACTB*; forward primer 5′-TGG-TGA-TGG -AGG-AGG-TTT-AGT-AAG-T-3′; reverse primer 5′-AAC-CAA-TAA-AAC-CTA-CTC-CTC-CCT-TAA-′3). Relative DNA methylation levels were calculated using the delta-delta-CT formula.

2.5. Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) protocol was adopted from Shang et al. [12]. In brief, 4×10^6 cells were crosslinked in 1% formaldehyde at 20 °C; crosslinking was stopped after 3 min by glycine at a final concentration of 0.125 M. Cells were washed twice with cold *PBS* (PAA Laboratories) and resolved in 200 μl lysis buffer (0.1% SDS, 50 nM Tris–HCl, 10 mM EDTA) and incubated for 10 min on ice at pH 8.1. Chromatin was sheared by sonication using a Bioruptor (Diagenode, Liège, Belgium; setting: 30 s on/off for 10 min), followed by centrifugation for 10 min at 4 °C and collecting of the supernatants. Successful sonication (DNA fragmentation to approximately 100 bp) was controlled via agarose gel electrophoresis. Antibodies against H3K4me2 (catalog No. #07-030; Millipore, Billerica, MA, USA), H3K4me3 (#07-473; Millipore), H3K9me2 (#39239, Active Motif, Carlsbad, CA, USA), H3K9me3 (#07-442; Millipore), H3K27me2 (#07-452, Millipore), H3K27me3 (#CS-069-100; Diagenode), H3K18ac (#07-354, Millipore) or IgG negative-control (#33133, Santa Cruz, Santa Cruz, MA, USA) were pre-incubated with Magnetic Dynabead Protein A (Invitrogen). The dynabeads facilitated washing and elution of the immunoprecipitates by magnetic separation. The sonicated samples were incubated with a protein inhibitor (Complete25X, Hoffmann LaRocheAG, Basel, Switzerland), salmon sperm DNA (Stratagene, La Jolla, CA, USA) and the dynabead–antibody complex overnight for immunoprecipitation. Afterwards, the antibody–bead-complex was washed (10 nM Tris–HCl, 1 mM EDTA, 0.01% Tween20; pH 8.0) and eluted (elution buffer: 20 nM Tris–HCl, 5 nM EDTA, 50 nM NaCl, 1% SDS, pH 7.5). DNA was heated overnight (65 °C) for reverse-crosslinking. Finally, the DNA was purified by ethanol–ammoniumacetate immunoprecipitation. Reagents not otherwise specified were purchased from Merck, Darmstadt, Germany.

The amount of a specific histone modification at the *GSTP1* (primer sequences: forward 5′-TAT-AAG-GCT-CGG-AGG-CCG-3′; reverse 5′-GCG-TAC-TCA-CTG-GTG-GCG-A-′3) promoter was determined using real-time PCR on an ABI PRISM *7900HT* using SYBR GreenER qPCR Supermix (Invitrogen). The relative enrichment of each histone modification was calculated using the delta-delta-CT formula; normalization was performed against the 1:100 diluted input DNA.

3. Results

3.1. Induction of apoptosis

BPH-1, PC-3 and LNCaP cell lines were treated with depispeptide at a dosage of 5 nM for 48 h. The dosage and time were assessed by preliminary experiments and review of literature. Cell viability was reduced to ~20% after 48 h treatment with depsipeptide. As demonstrated in Fig. 1, treatment with 5 nM depsipeptide resulted in an increase of apoptotic cells (sub-G1 peak) after 48 h. There was no evidence of a cell cycle arrest.

3.2. Induction of GSTP1 DNA demethylation and mRNA re-expression

First, we confirmed the methylation status at the promoter CpG island in LNCaP (methylated), PC-3 (partially methylated) and BPH-1 (unmethylated) cells. Nearly undetectable levels of *GSTP1* mRNA were observed in LNCaP cells, and *GSTP1* expression was weak in PC-3 cells (data not shown). Treatment with 5 nM depsipeptide increased the *GSTP1* mRNA expression in PC-3 and LNCaP cells, whereas its expression was unchanged in BPH-1 cells. The changes of mRNA expression were accompanied by demethylation in both cell lines (Fig. 2).