Involvement of MyoD and PEA3 in regulation of transcription activity of MDR1 gene

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Overexpression of multidrug resistance 1 (MDR1) in cancer remains one of the major causes for the failure of chemotherapy. In the present study, we found that MyoD and PEA3 could activate P-glycoprotein (P-gp) expression in SGC7901 cells. Knockdown of MyoD and PEA3 attenuated MDR1 expression and increased the sensitivity of multidrug resistant cancer cells to cytotoxic drugs that were transported by P-gp in SGC7901/VCR cells. MyoD or PEA3 could bind to the E-box and PEA3 sites on the MDR1 promoter and activate its transcription. The regulation of MDR1 expression by MyoD and PEA3 may provide potential ways to overcome MDR in cancer treatment.

Keywords multidrug resistance; MyoD; PEA3; MDR1

Introduction

Multidrug resistance (MDR) in cancers remains a major cause for the failure of chemotherapy. Proteins involved in MDR mechanisms are P-glycoprotein (P-gp), MDR-associated proteins, major vault protein/lung resistance-related protein, and breast cancer resistance protein [1,2]. One form of MDR is caused by overexpression of P-gp, the MDR1 gene product [3,4]. Overexpression of P-gp confers cancer cell resistance to a variety of chemotherapeutic drugs and constitutes one of the major obstacles to successful treatment of numerous types of malignancies. P-gp is an efflux pump that is known to confer resistance to a variety of clinically important antineoplastic agents, including doxorubicin, vincristine, taxanes, etoposide, teniposide, and actinomycin D. Many of these agents are used to treat cancers [5,6].

Expression of P-gp can be induced or repressed by a variety of chemical or physical insults, such as cytotoxic agents [7,8], heat shock [9], UV irradiation [10,11], cytokines [12,13], and transcriptional factors [14,15]. MyoD and PEA3 are transcription factors, and so far, there is no report as to whether MyoD and PEA3 can regulate MDR1 transcription.

The aim of this study was to explore the activity of MyoD and PEA3 on MDR1 transcription and to reveal the role of MyoD and PEA3 involved in MDR1-mediated drug resistance, which would be helpful to understand the MDR mechanism.

Materials and Methods

Cell lines, culture, and plasmids

The MDR cancer cell line, SGC7901/VCR, and the sensitive cell line, SGC7901, were purchased from Wuhan University Type Culture Collection (Wuhan, China). They were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. For SGC7901/VCR cell line, 0.8 μmol/l of vincristine was added into the medium for the maintenance of the MDR phenotype. Cells were checked routinely and found to be free of contamination by Mycoplasma or fungi. All the cell lines were discarded after 3 months and new lines were obtained from frozen stocks.

MDR1 promoter/luciferase construct pGL2-MDR1 (−1202 to +118) was kindly provided by Dr. Kathleen W. Scotto (The Cancer Institute of New Jersey, USA). PEA3 expression vector was kindly provided by Dr. Hassell (Department of Biology, McMaster University of
Canada, Canada). MyoD expression vector (pCMV-MyoD) was purchased from Origene Company (Rockville, USA).

**Real-time polymerase chain reaction**

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the fluorescence temperature cycler (Opticon, MJ Research, Waltham, USA) and the SYBR Green PCR Core Reagents kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Genes and primers are listed as follows: *MDR1*, forward primer 5′-AT ATCAGCAGCCCACATCAT-3′; reverse primer 5′-GAAG CACTGGGATGTCCGGT-3′; *MyoD*, forward primer 5′-GC TCCGACGGCATGATGG-3′; reverse primer 5′-TAAGC GCTGTGGGGAGG-3′; *PEA3*, forward primer 5′-CAGCT CAGCTTTCTTCTAGGTC-3′; reverse primer 5′-CCTCTC TGCTTATACCAGCAC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers are as follows: 5′-GCCAAAGGGTCATC ATCTC-3′ and 5′-GTAGAGGGCGGATGATGTTCT-3′. An initial incubation of 50°C for 2 min was followed by denaturing at 95°C for 10 s and then 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR products were detected by bound SYBR Green double-stranded DNA fluorescence, and the comparative threshold cycle (2-ΔΔCt) method was used to quantify the mRNA of these genes. All samples were tested in triplicate. Target gene expression was compared with the housekeeping gene *GAPDH*. After PCR, a melting curve was obtained and analyzed.

**Western blot analysis**

Cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride and lysed in mammalian protein extraction buffer (Pierce, Rockford, USA). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 g for 40 min at 4°C. Equal amounts (50 μg of protein) of cell lysates were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membrane. The membranes were blocked in 5% powered milk solution in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with anti-P-gp antibody C219 (Calbiochem, San Diego, USA), anti-MyoD and anti-PEA3 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, USA), or anti-tubulin antibody (Sigma-Aldrich, St Louis, USA), respectively. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, USA).

**Small interfering RNA preparation and transfection**

The small interfering RNA (siRNA) targeting MyoD and non-targeting siRNA were purchased from Santa Cruz Biotechnology, Inc. The siRNA targeting PEA3 was purchased from Ambion (Austin, USA). Cells in the exponential phase of growth were seeded in six-well plates at a concentration of 5 × 10⁵ cells/well. After incubation for 24 h, cells were transfected with siRNA specific for MyoD and PEA3 or non-targeting siRNA at a final concentration of 100 nM using oligofectamine and OPTI-MEMI reduced serum medium (Invitrogen), according to the manufacturer’s protocol. Silencing was examined at 48 h after transfection.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChiP) assays were carried out according to the manufacturer’s protocol (Active motif, Carlsbad, USA). Briefly, cells in 150-mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37°C. The cells were then washed twice with ice-cold PBS, harvested, and resuspended in ice-cold TNT lysis buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 1% aprotinin). The lysates were sonicated to shear the DNA into fragments of 200–600 bp, and then subject to immunoprecipitation with the following antibodies, respectively, anti-MyoD and anti-PEA3 or immunoglobulin G (Santa Cruz Biotechnology, Inc.) as a negative control. Three micrograms of antibody was used for each immunoprecipitation. The antibody/protein complexes were collected by Protein G beads and washed three times with ChIP wash buffer [5% SDS, 1 mM EDTA, 0.5% bovine serum albumin, and 40 mM NaHPO4, pH 7.2]. The immune complexes were eluted with 1% SDS and 1 M NaHCO3, and the cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and DNA was purified by mini-column, ethanol precipitation, and resuspended in 100 μl of H2O. The primers corresponding to the *MDR1* promoter region −164 and −11 upstream of the transcription start site (sense: 5′-CCTCTGGAAAATTCGAC CG-3′; antisense: 5′-TGTGGCAAAGAGGAGCGAAG-3′) was used for real-time PCR or RT–PCR to detect the presence of the *MDR1* promoter DNA.

**Mutagenesis**

*MDR1* promoter/luciferase construct pGL2-MDR1 was used as template. The plasmid DNA was methylated with DNA methylase at 37°C for 1 h. The plasmid was amplified in a mutagenesis reaction with two overlapping primers, one of which contains the target mutation. The product was linear, double-stranded DNA containing the mutation. The mutagenesis mixture was transformed into wild-type *Escherichia coli*. The host cells circularized the linear mutated DNA, and McrBC endonuclease in the host cells could digest the methylated template DNA, only leaving the unmethylated and mutated product. For
individual mutations, the sequence of MyoD-binding site (E-box-2) CATCTG was converted to AAATTT; non-canonical E-box-1 and E-box-3 (CACAGG and CAACGG) were also converted to AAATTT; and PEA3-binding site (PEA3-1, PEA3-2 and PEA3-3) AGGAA was converted to ATATG.

**Luciferase reporter gene assay**
SGC7901 cells were seeded in 6-well plates at a density of 1–2 × 10^5 cells/well and cultured for 24 h. Cells were then co-transfected, wild-type (pMDR1) or mutant (E-box mutant or PEA3 mutant) MDR1 reporter construct (0.5 µg/well), with 0.5 µg of pcDNA3, or CBP, p300, MyoD, PEA3 expression vector, respectively, together with 20 ng of control Renilla luciferase reporter construct pRL-TK (Promega, Madison, WI). The total amount of DNA per well was adjusted to 1.5 µg by the addition of sonicated salmon sperm DNA. Luciferase assays were performed as recommended by the vendor (Promega) and normalized relative to protein concentration as determined by the bicinchoninic acid protein assay (Pierce). The promoter activity was then expressed as luminescence units, which is the ratio of luminescence counts of cell lysate and the absorbance at 595 nm for the same amount of cell lysate stained with bicinchoninic acid protein assay reagent.

**Drug sensitivity assay**
SGC7901/VCR cells transfected with siRNA targeting MyoD and PEA3 or non-targeting siRNA (control siRNA) were plated in 96-well plates in growth medium and incubated at 37°C in a humidified 5% CO2 atmosphere for 60 h in the presence of varying concentrations of vincristine, doxorubicin, paclitaxel, and cisplatin. The viability of cells was measured by MTT Assay.

**Statistical analysis**
Analysis of variance and Student’s t-test were used to determine the statistical significance of differences between experimental groups. P-values of <0.05 were considered significant, and the confidence intervals quoted were at the 95% level.

**Results**

**Binding status of MyoD complex or PEA3 on the MDR1 promoter between SGC7901 and SGC7901/VCR cells**
In our experiments, we found that MDR1 transcription and P-gp expression were low in SGC7901 cells, and MDR1 transcription and P-gp expression were activated in SGC7901/VCR cells [Fig. 1(A,B)]. In order to determine the potential transcriptional factors binding to the MDR1 promoter, SGC7901 and SGC7901/VCR cells were subject to ChIP assays. ChIP results demonstrated that MyoD and PEA3 could bind directly to the transcriptional MDR1 promoter in SGC7901/VCR cells [Fig. 1(C,D)].

**Overexpression of MyoD or PEA3 increases P-gp expression in SGC7901 cells**
To identify the role of MyoD or PEA3 in regulating the expression of P-gp, we transfected MyoD or PEA3 expression vector into SGC7901 cells, respectively. Figure 2(A) showed that overexpression of MyoD could increase P-gp expression in SGC7901 cells. Figure 2(B) showed that overexpression of PEA3 could increase P-gp expression in SGC7901 cells.

**Effect of MyoD or PEA3 on the MDR1 promoter activity**
To identify the role of MyoD or PEA3 in regulating MDR1 promoter transcription, we co-transfected the MDR1 promoter/luciferase construct pMDR1 with pcDNA3, MyoD, or PEA3 expression vectors into SGC7901 cells, respectively. Figure 3(B) showed that the luciferase activities were enhanced significantly by MyoD or PEA3, indicating that MyoD and PEA3 were involved in the activation of the MDR1 promoter activity.

As shown in Table 1 and Fig. 3(A), we found three E-box sites from −200 to +1 bp on the MDR1 promoter. To determine the potential roles of these E-box elements in regulation of MDR1 gene transcription, we individually and combinatorially mutated the three E-box sites (E-box-1, E-box-2, and E-box-3) close to the transcriptional starting site on the MDR1 promoter and examined MyoD-inducible reporter gene activities in SGC7901 cells.

As shown in Fig. 3(C), any single mutation of three E-box sites decreased the reporter gene activity compared with wild-type MDR1 promoter construct when cotransfected with MyoD. Mutations of any two E-box sites simultaneously caused a further decrease in reporter gene activity, and the combined mutations of all three E-box sites resulted in the maximal decrease in reporter gene activity. These results suggested that all three E-box sites contribute in a concerted mechanism to the MyoD-induced transcription of MDR1 gene, and MyoD activates MDR1 transcription by binding to a number of E-box sites on the MDR1 promoter.

We also found four PEA3 sites from −200 to +1 bp on the MDR1 promoter [Table 1 and Fig. 3(A)]. To determine the potential roles of these PEA3 elements in regulation of MDR1 gene transcription, we individually and combinatorially mutated the three PEA3 sites (PEA3-1, PEA3-2, and PEA3-3) close to the transcriptional starting site on the MDR1 promoter and examined PEA3-inducible reporter gene activities in SGC7901 cells.
As shown in Fig. 3(D), any single mutation of three PEA3 sites decreased the reporter gene activity compared with wild-type MDR1 promoter construct when cotransfected with PEA3. Mutations of any two PEA3 sites simultaneously caused a further decrease in reporter gene activity, and the combined mutations of all three PEA3 sites resulted in the maximal decrease in reporter gene activity. These results suggested that all three PEA3 sites contribute in a concerted mechanism to the PEA3-induced transcription of MDR1 gene, and PEA3 can activate MDR1 transcription by binding to a number of PEA3 sites on the MDR1 promoter.

Knockdown of MyoD and PEA3 attenuates MDR1 expression and recruitment of MyoD and PEA3 on the MDR1 promoter

SGC7901/VCR cells were treated with 100 nM of MyoD and PEA3 siRNA or non-targeting siRNA for 48 h and performed real-time PCR and western blot analysis. As shown in Fig. 4(A,B), MyoD and PEA3 siRNA significantly inhibited MyoD and PEA3 mRNA and protein in SGC7901/VCR cells after transfection for 48 h. At the...
same time, we found that knockdown of MyoD and PEA3 inhibited MDR1 mRNA and protein significantly. Then, we transfected MyoD [Fig. 4(C,D)] or PEA3 siRNA [Fig. 4(E,F)], respectively, into SGC7901/VCR cells and found that either of them could inhibit MDR1 mRNA and P-gp protein; however, it was not as significantly as that in cells transfected with MyoD and PEA3 siRNA together.

ChIP results demonstrated that knockdown of MyoD and PEA3 inhibited the recruitment of MyoD and PEA3 on the MDR1 promoter [Fig. 5(A,B)].

**Knockdown of MyoD and PEA3 can modulate MDR phenotype**

We then tested whether knockdown of MyoD and PEA3 could increase the sensitivity of MDR cancer cells to cytotoxic drugs that were transported by P-gp. SGC7901/VCR cells were transfected with siRNA targeting MyoD and PEA3 or non-targeting siRNA (Control siRNA) for 6 h, and then were seeded in 96-well plates and incubated in the presence of various concentrations of doxorubicin, paclitaxel, vincristine, or cisplatin for 54 h. As shown in **Table 2**, compared with control MDR cells, the sensitivity of MDR cells transfected with MyoD and PEA3 siRNA to doxorubicin, vincristine, and paclitaxel was increased 2.38-, 2.42-, and 2.62-fold, respectively. However, the sensitivity to cisplatin, a drug that is not transported by P-gp, was not affected by silencing of MyoD and PEA3 expression.

**Discussion**

The basic helix-loop-helix transcription factor, MyoD, induces differentiation of myoblasts into myotubes by transcriptional activation of downstream target genes. MyoD is active in dividing cells. Inhibitor of DNA binding 3 and neuronal pentraxin 1 are two direct transcriptional targets of MyoD. MyoD functions as a transcriptional activator in myogenic stem cells [16]. MyoD interacts with estrogen receptor α, histone acetyltransferase CBP and p300, and RNA helicases p68 and p72 and forms an activating
transcriptional complex that can bind to many Sp1 sites on the BRCA2 promoter and activate its transcription [17]. MyoD interacts directly with both p300/CBP and p300/CBP-associated factor, forming a multimeric protein complex on the promoter elements for the activation of myogenic program [18]. Consensus sequence for Myod binding is CANNTG (E-box) [19]. The results from serial deletions of the VEGF promoter elucidate that the region containing three E-box sites is essential for MyoD-mediated up-regulation of VEGF [20].

The PEA3 subfamily includes PEA3, ER81, and ERM [21–23]. PEA3 may play a role in human breast cancer. The human PEA3 gene is up-regulated in breast tumor cell lines [24] and 93% of HER2/Neu-positive human breast tumors [25]. PEA3 expression is also found in the majority of both clinical specimens and lung and oral carcinoma cell lines [26–28]. PEA3 can regulate the transcription of several proteases, including the matrix metalloproteinases (MMP) collagenase-IV/gelatinase B (MMP-9), matrilysin (MMP-7), and stromelysin-3 (MMP-11), and the serine protease urokinase-type plasminogen activator [29,30]. However, transfection with an antisense sequence of PEA3 in oral carcinoma cells can result in inhibition of invasion and MMP expression [31]. Transfection with PEA3 results in enhanced motility and invasion in lung cancer cells and human SKBR3 breast cancer cells [26,30]. Expression of a PEA3 dominant-negative form reduces tumor growth in this model [32]. All these reports indicate that PEA3 is related to cancer motility and invasion.

We used SGC7901 and SGC7901/VCR cells in our research. SGC7901/VCR is an established VCR-resistant cell line selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR [33]. Previous reports have proved that P-gp expression is in a very low level in SGC7901 cells, but in a high level in SGC7901/VCR cells. The SGC7901/VCR cell line was 45 times more resistant to VCR than the parental SGC7901 cell line [34]. The SGC7901/VCR cell line has been successfully used as an in vitro MDR reversal model [35].
In this study, the recruitment of Myod or PEA3 increased significantly on the transcriptional MDR1 promoter, knockdown of MyoD and PEA3 could inhibit the recruitment of MyoD and PEA3 on the MDR1 promoter region in SGC7901/VCR cells, indicating MyoD and PEA3 might regulate MDR1 expression. Bioinformatic analysis of the S' flanking region of the human MDR1 gene showed that there exist four PEA3 sites and three E-box sites on the MDR1 promoter region from -200 to +1 bp, which indicated that PEA3 and Myod might bind to MDR1 promoter and regulate MDR1 transcription. Point mutant of E-box on the MDR1 promoter construct abrogated the activation effect of Myod on MDR1 promoter activity, indicating that Myod activated MDR1 promoter activity by directly binding to the E-box of MDR1 promoter. Point mutant of the PEA3 site in the MDR1 promoter construct abrogated the activation effect of PEA3 on MDR1 promoter activity, indicating that PEA3 activated MDR1 promoter activity by directly binding to the PEA3 site of MDR1 promoter.

In this study, we also found that overexpression of MyoD and PEA3 could increase P-gp expression and knockdown of MyoD and PEA3 could attenuate MDR1 expression and P-gp expression, which further confirmed that MyoD and PEA3 were responsible for P-gp expression. Treatment of MDR cells with MyoD and PEA3 siRNA enhanced the sensitivity of MDR cells to doxorubicin, paclitaxel, and vincristine, but had no effect on the sensitivity of MDR cells by influencing P-gp expression. We conclude that MyoD and PEA3 can activate MDR1 transcription. Knockdown of MyoD and PEA3 attenuates MDR1 expression and increases the sensitivity of MDR cancer cells to cytotoxic drugs that are transported by P-gp. MyoD or PEA3 can bind to E-box or PEA3 sites on the MDR1 promoter and activate its transcription. The elucidation of the role of MyoD and PEA3 in MDR1 gene transcription might lead to a better understanding in the regulation of MDR1 and help to develop new strategies to inhibit or prevent the induction of MDR1 expression.

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