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ORIGINAL ARTICLE

Inducible expression of antizyme 1 in prostate cancer cell lines after lentivirus mediated gene transfer

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Abstract The prostate has the highest level of polyamines among all tissues, and it is the only tissue in which polyamines are purposely synthesized for export. It has been suggested that the high local polyamine concentrations suppress cell growth of primary prostatic carcinomas and that this growth control is lost when cancer cells metastasize. It has also been shown that the sensitivity to polyamine-induced growth arrest correlates with antizyme induction in prostate carcinoma cell lines. In this study, we evaluated the sensitivity of poorly metastatic (LNCaP) and highly metastatic (DU145) prostate cancer cell lines to conditional antizyme 1 over-expression. Antizyme 1 induction resulted in a marked loss of ODC activity and polyamine uptake in both cell lines. However, the proliferation of LNCaP cells was repressed by antizyme 1 induction, whereas the proliferation of DU 145 cells was not affected. Neither cell line showed any reduction in polyamine pools after manipulation nor did polyamine addition into the medium save the LNCaP cells from the growth retardation. The growth inhibition of LNCaP cells was accompanied by accumulation of cells in the G1 phase and depletion of cyclin E1 protein. These results confirm that different prostate cancer cell lines show diverse sensitivities to antizyme 1 which may not be directly polyamine related. The high gene transfer capacity of the used lentiviral vector makes the present approach a useful tool to study the therapeutic potential of antizyme 1 both in cell cultures and experimental animals.

Keywords Cell cycle · Gene therapy · Ornithine decarboxylase · Polyamine

Abbreviations

AZ	Antizyme
AZIn	Antizyme inhibitor
DFMO	Difluoromethylornithine
LTR	Long terminal repeat
MOI	Multiplicity of infection
ODC	Ornithine decarboxylase
SIN	Self inactivating

Introduction

The incidence of prostate cancer has rapidly increased during the past 10 years and it is currently the most common male malignancy and the second most common cause of cancer deaths among elderly males. In spite of its high prevalence, molecular mechanisms of carcinogenesis and tumorigenesis are still poorly understood. Initially, prostate tumors respond to hormonal therapies, but in time, androgen-independent tumors emerge. In spite of androgen independence, the androgen signaling is intact in the majority of tumors and there are no mutations in the androgen receptor itself (Devlin and Mudryj 2009). This notation has turned the attention towards the pathways downstream from the androgen signaling. The polyamines, spermidine, spermine, and putrescine are organic cations absolutely required for cell growth, and prostate is the

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richest source of polyamines in the mammalian body (Schipper et al. 2003). Furthermore, their biosynthesis is under direct androgen regulation in prostate epithelia (Crozat et al. 1992). Induction of the biosynthetic enzyme of polyamines, ornithine decarboxylase (ODC), is also one of the early events in prostate carcinogenesis (Young et al. 2006), and polyamine biosynthesis is one of the most upregulated processes in prostate cancer samples (Rhodes et al. 2002). A recent clinical study demonstrated that reduction of prostate tumor size can be achieved by oral administration of an ODC inhibitor, difluoromethylornithine (DFMO) (Simoneau et al. 2008). Similarly, DFMO suppressed cancerous growth and prevented formation of metastases in the transgenic adenocarcinoma model of mouse prostate (TRAMP) (Gupta et al. 2000). However, the clinical trials for the treatment of advanced prostate cancer with the inhibitors of polyamine biosynthesis have been unsuccessful due to the compensatory activation of both biosynthesis and uptake of extracellular polyamines (Shantz and Levin 2007).

Antizymes are unique regulatory proteins which regulate both the polyamine biosynthesis and uptake (Kahana 2007), and thus provide an approach to control cellular proliferation, in situations were mere biosynthesis inhibition is not sufficient. Indeed, the ectopic expression of AZ1 has been shown to deplete cellular polyamines to a level which induces cell death and blocks tumor formation both in vitro and in vivo tumor models (Iwata et al. 1999; Feith et al. 2001). Of the three known mammalian antizymes, the antizyme 1 (AZ1) is the best known and ubiquitously expressed. Antizyme 2 is also ubiquitously expressed but to a lesser extent and is less efficient in targeting ODC to proteosomal degradation. Antizyme 3 is only expressed in testis. There are also two antizyme inhibitor proteins (AZIn) known to date, one ubiquitously expressed (AZIn1) and the other which is restricted to brain and testis (AZIn2). AZIn binds to antizymes and thus negates their effects on polyamine metabolism (Lopez-Contreras et al. 2010).

In this study, we used inducible over-expression system which was based on lentiviral vector developed by Shin et al. (2006). Lentiviruses belong to retroviruses and they can be used for an efficient delivery of transgenes into the genome of various cells types, independent of the proliferation state. The design of novel, more biosafe lentiviral vectors has enabled their routine use in experimental research, where high gene transfer efficiency is needed, e.g. in gene therapy studies and in transgenesis of experimental animals (Sinn et al. 2005). Here, we used lentiviral transgenesis to over-express AZ1 in cultured cells at levels that markedly reduced ODC activity. We also demonstrated that from the two prostate cancer cell lines, the proliferation of only LNCaP cells was sensitive to AZ1 induction and that this sensitivity was not accompanied by a reduction of polyamine levels.

Materials and methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) if not otherwise stated.

Transgenic cell lines

The human prostate cell lines were obtained from the American Type Culture Collection (Rockville, MD). LNCaP is an androgen responsive, prostate-specific antigen expressing, prostate carcinoma and DU145 is an androgen unresponsive brain metastasis of a prostate carcinoma. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 10 mM Hepes, 1 mM Na-pyruvate, 2 mM glutamine and 50 μg/ml gentamycin. In experiments, where polyamines were added into the culture media, 1 mM aminoguanidine was used to prevent polyamine oxidation. Cells were cultured at 37°C in 95% air and 5% carbon dioxide. A mutant mouse AZ1 cDNA construct lacking T at the site 205 was used to obtain efficient AZ1 expression without the need for frameshifting (Kankare et al. 1997). For doxycycline inducible expression, a novel system containing a reverse tetracyline transactivator, the gene construct of choice under tetracycline responsive element, and a selection marker in a single lentiviral vector were used (Shin et al. 2006). The lentiviral particles were produced as described earlier (Shin et al. 2006). DU145 and LNCaP cells were infected at MOI 1 and subjected to neomycin selection (0.5 mg/mL Geneticin) for 2 weeks. The lowest concentration of doxycycline which maximally induced antizyme was experimentally determined to be 250 ng/ml, a concentration which was used thereafter.

Analytical methods

Western blotting was performed according to a laboratory manual, Current Protocols in Molecular Biology (Ausubel et al. 2007). Shortly, 25 μg of protein was used for SDS–PAGE gel electrophoresis and transferred onto the PVDF membranes. Membranes were blocked with 2% ECL advance blocking reagent (GE Healthcare, Fairfield, CT, USA) in phosphate-buffered saline containing 0.1% tween and probed with 1:1,000 dilution of Cyclin A, B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), D1 (Thermo Fisher Scientific, Fermont, CA, USA), E1 (Abcam, Cambridge, UK), β-actin (Santa Cruz Biotechnology) Antizyme 1 [described in Kankare et al. (1997)], and Antizyme inhibitor 1 (Cosmo Bio, Tokyo, Japan)

Table 1 Polyamine uptake, ODC activities and polyamine pools after AZ induction (+Dox) or after ODC inhibition by DFMO

Cell line + treatment	Polyamine uptake (pmol/min/10^6cells)	ODC activity (pmol/h/10^6cells)	Putrescine	Spermidine (pmol/10^6 cells)	Spermine
Du 145	14.6 ± 0.70	1,250 ± 199	125 ± 16.6	1,460 ± 85.7	928 ± 57.4
+250 ng/ml Dox	8.94 ± 0.34*	313 ± 63.5*	104 ± 31.2	1,410 ± 61.8	1,080 ± 27.4*
+1 mM DFMO	n.d.	64.8 ± 17.1*	<50	<50*	1,440 ± 50.4*
LNCaP	4.30 ± 0.35	192 ± 30.3	<50	784 ± 207	3,270 ± 90.4
+250 ng/ml Dox	1.83 ± 0.05*	32.0 ± 6.3*	<50	1,250 ± 397	3,610 ± 424
+1 mM DFMO	n.d.	222 ± 15.3	<50	406 ± 104	3,590 ± 187

The ODC activity was measured at the active growth phase (2 days time point) and the polyamines was measured at the end of the experiment (5 days time point). A separate experiment was performed to study polyamine uptake. The results are expressed as means ± SD from three measurements. * $p < 0.01$ versus untreated. *n.d.* not determined

antibodies. DyLight 680 and 800-conjugated secondary antibodies (Thermo Fisher Scientific) were used in 1:10,000 dilutions. Antibody-bound protein was detected by the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Polyamines were quantified using high-performance liquid chromatography as described earlier (Hyvönen et al. 1992). The activity of ODC was assayed by the method described in Jänne and Williams-Ashman (1971).

For uptake experiments, the cells were plated onto six-well plates at a density of 0.2×10^6 cells/well and incubated for 48 h in the presence or absence of doxycycline (250 ng/mL). The medium was changed to a labeling medium containing 0.25 µCi/ml [^{14}C] -spermidine (GE Healthcare) and 10 µM spermidine in RPMI-1640 without serum and incubated 10 min before harvesting. The cells were washed twice with ice-cold PBS and the incorporated radioactivity was detected with a liquid scintillation counter.

For cell cycle analysis, the cells were determined for their DNA content by staining them with propidium iodide essentially as described by Prather et al. (1999). The stained cells were then run in a flow cytometer, detecting red fluorescence. Single parameter histograms (*X*-axis, red fluorescence and *Y*-axis, number of cells) were used to determine the proportion of different cell cycle phases in each population. The cells with haploid DNA content represented G0 + G1 phases, cells with diploid DNA content were at G2 + M phases and the cells with intermediate DNA content represented S-phase.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test. A p value of less than 0.01 was considered significant. Data are expressed as means ± standard deviation.

Results

The transgene construct proved to be functional as the expression of AZ1 could be tightly regulated by the application of doxycycline. The maximum expression of AZ1 was achieved at the concentration of 250 ng/ml of doxycycline and the expression reached the maximum after 4 h of treatment and remained stable thereafter (not shown). The over-expressed AZ1 protein was also rapidly degraded ($t_{1/2} \approx 0.5$ h) when its production was blockaded by 10 µg/ml cycloheximide treatment (not shown). The over-expressed AZ1 was functional, as it down-regulated both the ODC activity (Table 1) and polyamine uptake (Table 1). The induction of AZ1 also evoked a compensatory up-regulation of AZIn1 protein in LNCaP cells (Fig. 1).

AZ1 induction repressed the proliferation of LNCaP cells and this could not be prevented by external polyamines (Fig. 2). The growth of DU145 was not affected by AZ1 induction. Regardless of the markedly reduced biosynthesis and uptake of polyamines, there was no reduction of the polyamine pools in either of the cell lines (Table 1). These results were opposite to the effect of the specific ODC inhibitor, DFMO, which inhibited the proliferation of DU 145 cells efficiently by depleting the level of spermidine. On the other hand, this concentration of DFMO (1 mM) was not efficient enough to inhibit the ODC activity or the proliferation of LNCaP cells (Table 1, Fig. 2).

To pinpoint the cell cycle traverse being most severely affected in LNCaP cells with induced AZ1, we analyzed the cell cycle phases by FACS analysis and by detection of the cyclin proteins. A prolonged 4 day AZ1 induction increased the proportion of cells in G0 + G1 from 66 to 78%. The most affected cyclin was E1 which almost disappeared after the second day of AZ1 induction (Fig. 3). Also cyclins A and B1 were affected but to a lesser extent.

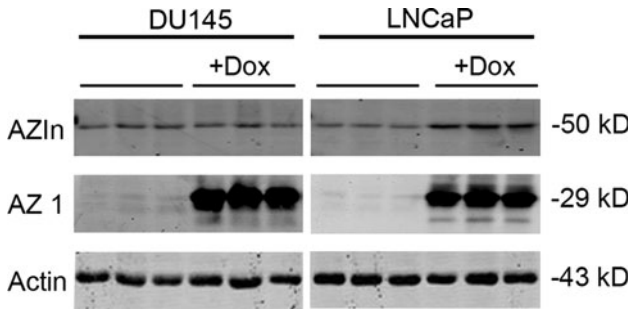

Fig. 1 Compensatory upregulation of AZIn1 in LNCaP cells in response to AZ1 induction. The AZ1 expression was induced by treating the transgenic cells with 250 ng/ml Dox for 24 h. Actin protein was used as loading control

Interestingly, the level of cyclin D1 was virtually intact, even though its proteosomal degradation has been shown to be partly regulated by antizyme (Newman et al. 2004).

Discussion

In this study, we produced lentiviral particles which can efficiently be used to evoke long-term inducible AZ1 over-expression in cell cultures or in tissues of living animals. The lentiviral vector was self-inactivating (SIN) to minimize the risk of the promoter interference due to viral LTRs and of expression of viral components (Miyoshi et al. 1998). The used approach is particularly well suited for experimental gene therapy applications, as the expression of AZ1 can be regulated by a well-tolerated oral drug, doxycyclin. The functionality of the system was tested on Du145 and LNCaP prostate carcinoma cell lines, which have been previously shown to have diverse sensitivities to polyamines and their analogues (Mi et al. 1998). It has later been demonstrated that the sensitivity of different prostate carcinoma cell lines to polyamines correlate with antizyme induction (Koike et al. 1999). Indeed, the two chosen cell lines responded differently as the growth of the highly metastatic DU145 cell line was not affected by AZI induction, whereas, the growth of the poorly metastatic, androgen-responsive LNCaP cell line showed severe growth inhibition.

It is paradoxical that the AZ1 over-expressing LNCaP cells were able to maintain their normal polyamine pool after prolonged inhibition of both the polyamine synthesis and uptake. However, the former can be explained by polyamine-independent cytostasis occurring before the dilution of polyamines by cell division. The results showing that external polyamines were not able to rescue the cells from growth inhibition support the assumption. However, one must remember that the analysis of cellular polyamines gives little information on the availability of free polyamines for cellular functions, as a major part of polyamines is tightly bound to cellular macromolecules (Igarashi and Kashiwagi 2010), and even less information on the spatial distribution of polyamines is available. Therefore, it is possible that some vital functions in a specific cellular compartment suffer from polyamine depletion even when there is no measurable decrease in the total polyamine levels.

Interestingly, the two cell lines showed opposite growth responses to AZ1 over-expression and DFMO treatment. DU145 cells were very sensitive to DFMO and the depletion of ODC activity was accompanied by depletion of putrescine and spermidine (Table 1). In contrast, similar DFMO treatment only moderately inhibited LNCaP cell growth without significant reduction of ODC activity or polyamine concentrations. The AZ1 induction in DU145 cells did not result in almost complete inhibition of ODC activity as it did in LNCaP cells, which may partly explain why the two cell lines had diverse growth response to AZ1 over-expression. However, AZ1 induction may also regulate cell growth independent of ODC and polyamines, as there is growing evidence that antizymes regulate the degradation of variety of growth factors (Kahana 2009).

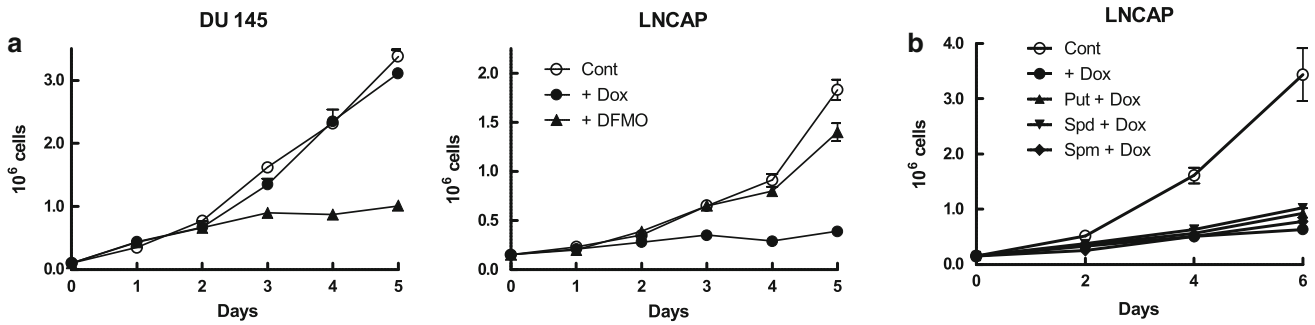

Fig. 2 Growth rates of transgenic prostate carcinoma cell lines. **a** Induction of AZ1 expression with 250 ng/ml Dox and inhibition of ODC with 1 mM DFMO resulted opposite effects on the proliferation of DU145 and LNCaP cell lines. **b** In a separate experiment the growth retardation of AZ1 induced LNCaP cells could not be prevented by the addition of 10 μM polyamines

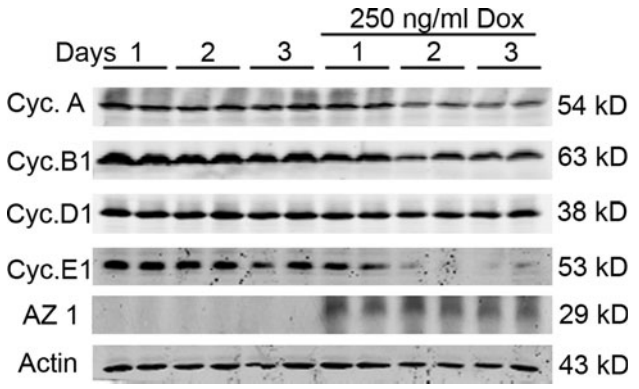

Fig. 3 Expression of cyclin E1 protein in LNCaP cells nearly disappears after second day of AZ1 induction. Transgenic LNCaP cells were cultured with and without 250 ng/ml Dox for 3 days. The expression of cyclins and AZ1 proteins was analyzed at each day. Actin protein was used as loading control

There was a clear increase on the proportion of LNCaP cells in G0 + G1 phase of the cell cycle after prolonged AZ1 induction. The effect of AZ1 over-expression seemed to be cytostatic rather than cytotoxic, as the proportion of apoptotic or necrotic cells did not increase significantly after AZ1 induction. Cyclin D1 is driving cells through the G1 phase and its proteosomal degradation is partly regulated by antizymes (Newman et al. 2004). Therefore, we analyzed the amount of cyclin D1 and other cyclin proteins after AZ1 induction. Interestingly, instead of cyclin D1, the most affected cyclin was E1 which is crucial to the transition of cells from G1 to S phase. Also the polyamine biosynthesis has been shown to peak at the G1/S transition (Alm and Oredsson 2009). It is thus tempting to speculate that this phase of the cell cycle is particularly sensitive to AZ1 action.

DU145 cells display a complete loss of tumour suppressor p53 function, while in LNCaP cells it is still functional. Mutations on p53 protein are known to result in defective cell cycle checkpoint control and are quite common in prostate cancers (Lee et al. 2008). The p53 exerts its inhibitory effect on cell cycle by inducing the transcription of p21 which in turn binds and inactivates the complexes of cyclins E, A and B and their partners cyclin-dependent kinases 1 and 2. Thus, the status of the p53 may well dictate the sensitivity of prostate carcinoma cell lines to AZ1 mediated G1 arrest. Indeed it has previously been shown that the status of p53 predicts whether polyamine analogue induces G1 arrest in melanoma cells (Kramer et al. 1999). Also in melanoma cells the G1 arrest resulted in clear decrease of cyclin E when the level of cyclin D1 even increased. However, the mechanistic details behind the diverse growth response to AZ1 induction remain to be determined. The used cell lines in this study were chosen according to their diverse responses to polyamine analogues. To unravel the mechanistic details, it might be more beneficial in future to choose closely related cell lines with known differences in their genome.

The conditionally inducible gene expression approach used here is invaluable in a situation where stable over-expression would result in undesirable effects. Inducibility also simplifies the experimental design as the control cultures are of the same origin as the induced ones, and the expression level can also be regulated by the concentration of doxycyclin. Here, we showed that AZ1 over-expression can be induced by doxycyclin dose-dependently. We also demonstrated that two different prostate cancer cell lines showed distinct responses to AZ1 induction and polyamine biosynthesis inhibition by DFMO treatment. These results support the idea that therapeutic approaches exploiting antizyme induction can be useful in situations where mere inhibition of polyamine biosynthesis is unsatisfactory.

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