The Wilms tumor suppressor gene *WT1* induces G1 arrest and apoptosis in myeloblastic leukemia M1 cells

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Abstract ***WT1*** **was isolated as a tumor suppressor gene of Wilms tumor. However, high expression of *WT1* correlates with poor prognosis in acute leukemia. In addition suppression of *WT1* expression by *WT1* anti-sense oligonucleotide inhibits proliferation of leukemia cells, suggesting that *WT1* is important for their proliferation. To further elucidate the biological significance of *WT1* in leukemic cell growth, we overexpressed exogenous *WT1* in murine M1 myeloblastic leukemia cells using the isopropyl-β-D-thiogalactoside (IPTG)-controlled expression system. We found that induction of one splicing variant of *WT1* [*WT1-17AA(+)-KTS(−)*] in M1 cells induces cell cycle arrest and apoptotic cell death. These results suggest that the role of *WT1* is different depending on the type of leukemia cell in which it is expressed.**

Key words: Tumor suppressor; WT1; Leukemia/cell cycle; G1 arrest/apoptosis

1. Introduction

Wilms tumor is a childhood kidney tumor that arises from metanephric blastema and is associated with inactivation of both alleles of the *WT1* gene [1–3]. *WT1* was isolated by the positional cloning technique and shown to be mutated in about 10% of sporadic Wilms tumors [2–6]. The *WT1* gene encodes a transcription factor with four carboxyl Cys^2–His^2 zinc fingers and two alternative splicing events, referred to as splices I and II, produce four isoforms of the protein [2,7]. Splice I results in the insertion of 17 amino acids upstream of the zinc finger domain and enhances the transcriptional activity of the protein [8]. Splice II occurs within the zinc finger domain, inserting three amino acids (Lys–Thr–Ser; KTS) between the third and fourth zinc fingers and changes the DNA binding specificity of the protein [9,10]. The WT1 protein without splice II specifically binds to the EGR-1 consensus sequence, to the (TCC)n motif and to the WTE motif [9,11,12]. By binding to these sites, the latter WT1 isoform can either repress or activate transcription from promoters of genes encoding insulin-like growth factor (IGF)-2, platelet-derived growth factor A (PDGF-A), insulin-like growth factor-1 receptor (IGF1R), epidermal growth factor receptor (EGFR), Bcl-2, c-Myc [13–16].

The *WT1* gene is expressed in various tissues including fetal kidney, testis, ovary and supportive structures of mesoderm origin [2,3,17–19]. Analysis of mice with homozygous deletion of the *WT1* gene has demonstrated the crucial role of this gene in early urogenital development [20]. The expression of *WT1* is also observed in hematopoietic tissues such as $CD34^+$ bone marrow cells and immature leukemia cells [21–23]. In some leukemia cell lines, down-regulation of the *WT1* gene occurs during differentiation along the myeloid and erythroid lineage pathways [24,25], and *WT1* antisense oligonucleotides inhibit the proliferation of these cells [26]. Interestingly, there is a correlation between high *WT1* expression and poor prognosis in acute leukemia [22]. These findings suggest that *WT1* is important for the malignant proliferation of leukemic cells.

In this study, we tried to further establish the biological significance of *WT1* in leukemic cell growth using M1 myeloblastic leukemia cells and the IPTG-inducible LacSwitch expression system to regulate expression of WT1. M1 cells are well known to be induced to differentiate in response to IL-6 [27,28]. We therefore imagined that exogenous expression of WT1 in M1 cells, which express barely detectable levels of WT1, may induce enhanced proliferation and resistance to the induction of differentiation. However, we unexpectedly found that the expression of exogenous WT1 containing splice I but not splice II in M1 cells induces cell cycle arrest and apoptotic cell death.

2. Materials and methods

2.1. Cell culture

Murine M1 myeloblastic leukemia cells were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and twice the standard concentration of amino acids and vitamins. Cells were transfected with the WT1 expression plasmids by electroporation. Drug-resistant colonies were selected by growth in G418 (450 μg/ml) or hygromycin (80 μg/ml). To generate growth curves, cells were seeded in 6 cm dishes at 5×10^4 cells/plate in the presence or absence of 5 mM IPTG and duplicate plates were counted. Viable cell numbers were determined by trypan blue exclusion and counted in a hemacytometer.

2.2. Plasmid construction and electroporation

The LacSwitch-inducible mammalian expression system (STRATAGENE) was used for inducible expression of WT1. cDNAs encoding various full-length human WT1 variants were cloned into pOPRSVI, an expression vector containing both RSV and lac operator sequences in its promoter. The WT1 constructs contained either alternative splice I alone or alternative splices I and II.

For electroporation, 10^7 cells were washed twice with PBS and resuspended in 0.8 ml of PBS containing 20 μg of p3′SS, a eukaryotic lac-repressor-expressing vector, and 20 μg of each pOPRSVI-WT1 construct and electroporated at 300 V, 960 μF using a Bio-Rad Gene pluser. Geneticin and hygromycin-resistant colonies were isolated and screened for WT1 expression by culturing in 5 mM IPTG followed by Western blot analysis.

2.3. Western blotting

Cell extracts for Western analysis were prepared from sub-confluent cultures, and 100 μg of protein from each cell line was analyzed by

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polyacrylamide gel electrophoresis. After transfer to an Immobilon-P membrane (Millipore, Bedford, MA), the blot was probed with monoclonal anti-WT1 antibody. The protein was detected using anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (Promega, Madison, WI). The anti-WT1 antibody recognizes both human and murine WT1.

2.4. DNA fragment assay

Cells were seeded at 4×10^5 cells per 6 cm dish, cultured in the presence or absence of 5 mM IPTG and harvested after 72 h. DNA was isolated and electrophoresed on a 2% agarose gel as described by Smith et al. [29].

2.5. Flow cytometric analysis

Cells (5×10^5 cells/dish) cultured in the presence or absence of 5 mM IPTG were harvested, washed, fixed and stained with propidium iodide. Flow cytometric analysis was performed on a FACScan (Becton Dickinson), using a ModFit LT.

3. Results

3.1. Inducible expression of exogenous WT1 in stably transfected M1 cells

To establish stable cell lines that express the exogenous *WT1* gene, we used an inducible expression system controlled by the lac-repressor. In this system, expression of the lac-repressor allows strict regulation of a promoter containing a lac operator. When *WT1* containing splices I and II [WT1-17AA(+)-KTS(+)] or *WT1* containing splice I but not splice II [WT1-17AA(+)-KTS(−)] was transfected into murine M1 myeloblastic leukemia cells, we obtained several cell lines in which exogenous *WT1* could be inducibly expressed in response to IPTG. Western blot analysis using monoclonal anti-WT1 antibody showed that *WT1* expression is undetectable in these cells in the absence of IPTG but increases after addition of IPTG (Fig. 1a). The induced protein was detected as early as 3 h after addition of IPTG (Fig. 1b).

3.2. Growth of cell lines expressing exogenous WT1

To examine the effect of expression of *WT1* on the growth of M1 cells, MD2 and MD8 cells, which inducibly express WT1-17AA(+)-KTS(−), and MB21 and MB27 cells, which inducibly express WT1-17AA(+)-KTS(+), were cultured in the presence or absence of IPTG and the number of viable cells were counted at various time points. The growth rate of MD8 (Fig. 2) and MD2 (data not shown) cultured in the absence of IPTG was virtually the same as that of the mock-transfected cells. However, in the presence of IPTG, the number of viable MD8 and MD2 cells increased for 72 h and started to decrease thereafter due to growth arrest and cell death. In contrast, the growth rate of MB27 (Fig. 2) and MB21 (data not shown) cultured in the presence of IPTG was the same as that of the mock-transfected cells. The effect of

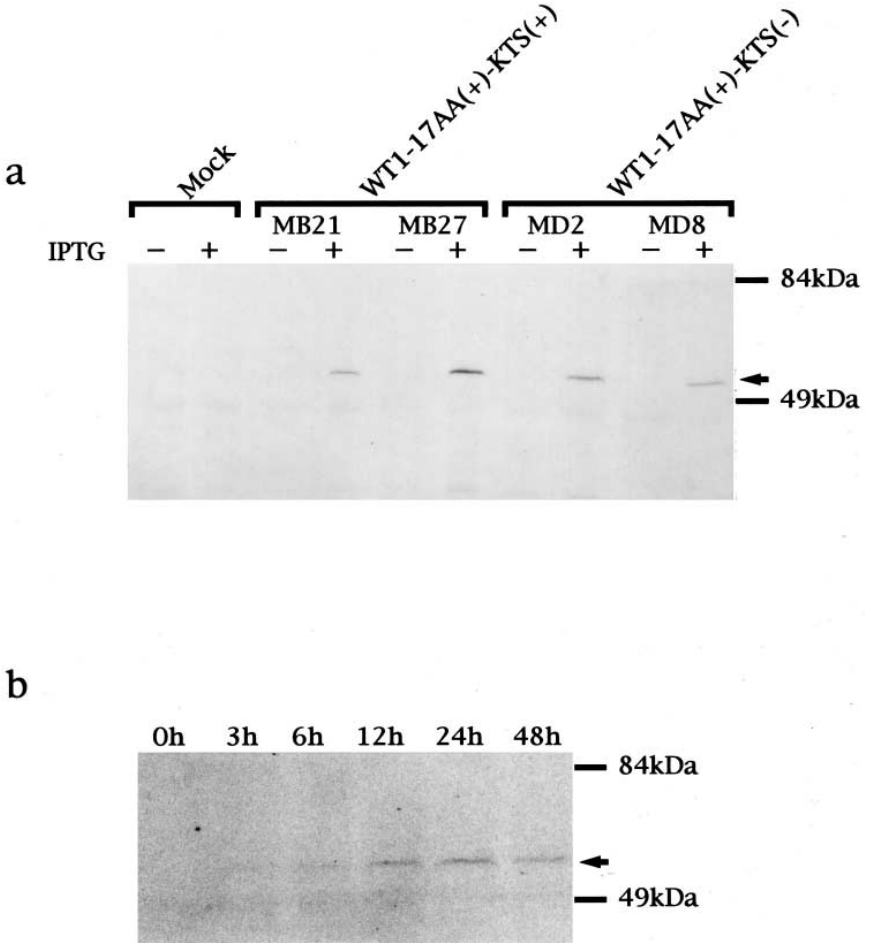

Fig. 1. a: Inducible expression of exogenous WT1 in M1-derived cell lines that had been transfected with *WT1-17AA(+)-KTS(+)* (MB21 and MB27) or *WT1-17AA(+)-KTS(−)* (MD2 and MD8). Lysates from M1 transfectants grown in the presence or absence of IPTG for 24 h were subjected to Western blot analysis using anti-WT1 monoclonal antibody. b: Time course of WT1 induction. MD8 cells were cultured in the presence of IPTG and harvested for Western blot analysis at the indicated time points. The arrow indicates WT1.

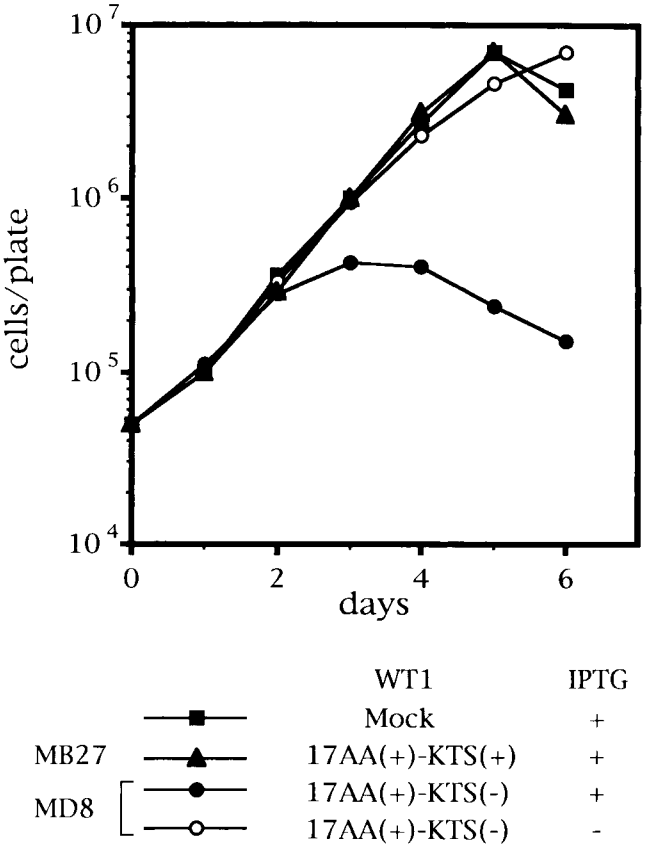

Fig. 2. Growth curves of cell lines expressing exogenous WT1. Cells were seeded in 6 cm dishes at 5.0×10^4 cells/plate and cultured for 6 days. Mock-transfected and MB27 cells were cultured in the presence of IPTG. MD8 cells was cultured in the presence or absence of IPTG. Viable cell number was determined by trypan blue exclusion at the indicated times.

WT1-17AA(+)-KTS(−) was observed regardless of the presence or absence of IL-6 in the medium (data not shown). On the other hand, WT1-17AA(+)-KTS(+) did not show the detectable effect on IL-6-induced differentiation of M1 cells (data not shown).

3.3. Cell cycle analysis of M1 cells expressing exogenous WT1

We previously demonstrated that overexpression of WT1 blocks cell cycle progression from the G1 to S phase in mouse fibroblast cells [30]. To test whether expression of WT1-17AA(+)-KTS(−) in M1 cells induces cell cycle arrest at a specific point, MD8 cells were subjected to cell cycle analysis after addition of IPTG. The relative percentage of cells in the G1 phase slowly increased from 35% up to 60% until 120 h after addition of IPTG (Table 1). However, in the absence of IPTG, MD8 cells exhibited little change in the percentage of cells in the G1 phase. MD2 cells also showed a quite similar cell cycle distribution pattern in response to IPTG (data not shown). These results suggest that expression of WT1-17AA(+)-KTS(−) in M1 cells induces G1 arrest.

Table 1
Cell cycle analysis of WT1-17A.A(+)-KTS(−)-expressing cells

	Cell cycle distribution (%)						
	0 h	24 h	48 h	72 h	96 h	120 h	
IPTG(−)	36.5	37.8	36.6	35.2	36.5	39.5	G1
	49.8	51.2	49.3	52.4	51.2	49.4	S
	13.7	11.0	14.1	12.4	12.3	11.1	G2/M
IPTG(+)	35.4	40.4	45.6	53.7	56.7	60.0	G1
	49.2	48.8	40.8	35.5	32.7	29.8	S
	15.4	10.8	13.6	10.8	10.6	10.2	G2/M

Percentage of MD8 cells in each cell cycle phase in the presence or absence of IPTG are shown (mean of two experiments).

3.4. Death of WT1-expressing M1 cells occurs by apoptosis

To examine whether WT1-induced cell death is caused by apoptosis or not, genomic DNA was prepared from MD8 and MB27 cells cultured in the presence or absence of IPTG. As shown in Fig. 3, genomic DNA from MD8 cells was fragmented into the typical nucleosome spacing ladder at 72 h after addition of IPTG. On the other hand, genomic DNA from MB27 and mock-transfected cells showed little or no indication of such DNA fragmentation. Similarly, MD2 but not MB21 showed DNA fragmentation when treated with IPTG (data not shown). These results suggest that expression of WT1-17AA(+)-KTS(−) induces apoptotic cell death.

4. Discussion

WT1 is highly expressed in many types of leukemia cells and high expression of WT1 correlates with poor prognosis in acute leukemia [21,22]. Furthermore, abrogation of *WT1* expression using antisense oligonucleotide inhibits proliferation of K562 cells and fresh leukemic cells from patients with AML and CML blast crisis, suggesting that WT1 is important for the proliferation of leukemic cells [26]. However, in the present study we show that overexpression of WT1-17AA(+)-

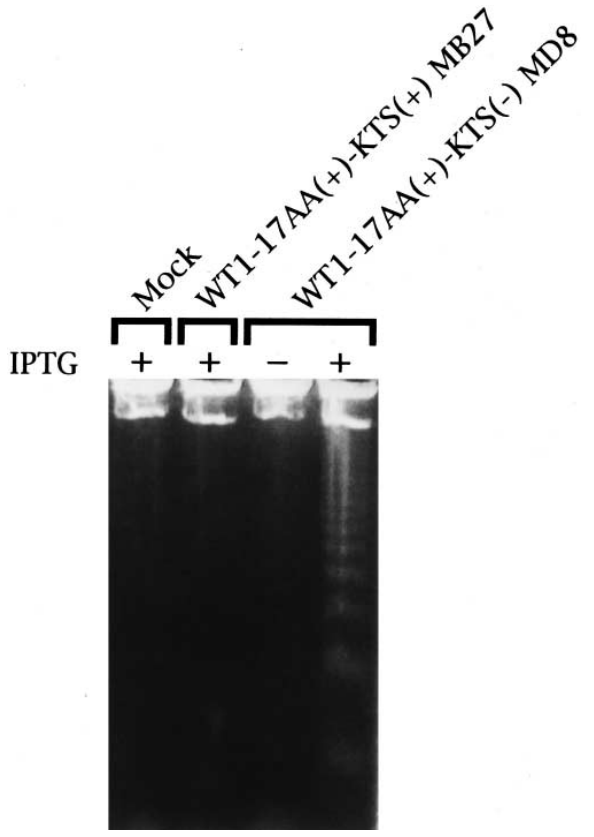

Fig. 3. DNA fragmentation assay. Cells were seeded in 6 cm dishes at 5.0×10^4 cells/plate and harvested after 72 h. Mock-transfected and MB27 cells were cultured in the presence of IPTG. MD8 cells were cultured in the presence or absence of IPTG. DNA was isolated and electrophoresed on a 2% agarose gel.

KTS(−) induces G1 arrest and apoptotic cell death in M1 myeloblastic leukemia cells. Thus the role of WT1 is different depending on the type of leukemia cell in which it is expressed. In this regard, it is interesting to note that WT1 shows different transcriptional activity depending on the type of cell. For example, WT1 suppresses transcription from the EGR1 promoter in NIH 3T3 and 293 cells in transient transcription assays [31], but activates it in Saos-2 cells and in cells expressing mutant p53 [32]. Hence, depending on the type of leukemia cell WT1 may differently regulate the transcription of genes which regulate cell growth and apoptotic cell death.

In cells derived from other tissues, *WT1* has been shown to have a potential to induce growth arrest and apoptosis. Overexpression of *WT1* blocks the G1-S transition of NIH 3T3 cells [30], while constitutive expression of *WT1* in F9 embryonal carcinoma cells induces apoptosis in response to retinoic acid [33]. WT1 also induces apoptotic cell death when ectopically expressed in osteosarcoma cell lines [15]. These findings also indicate that the effect of WT1 is different depending on the background of cells in which it is expressed.

While exogenous expression of WT1-17AA(+)-KTS(−) induced G1 arrest and apoptotic cell death, WT1-17AA(+)-KTS(+) did not show this activity. This difference may be partly explained by difference in transcriptional regulator activity of the two isoforms. The presence of KTS between zinc finger 3 and 4 changes the DNA binding specificity of the protein [12,34], and only WT1-17AA(+)-KTS(−) may be able to bind promoters of target genes critical for G1 arrest and apoptosis. In addition, it has recently been reported that the nuclear localization pattern of WT1-KTS(+) is different from that of WT1-KTS(−), and the former is found associated with splicing factors, suggesting that WT1-KTS(+) is involved in RNA metabolism [35]. Thus our data indicates that the putative post-transcriptional processing activity of WT1 is not important for the induction of G1 arrest and apoptosis.

WT1 has been reported to repress transcription of the *bcl-2* and *IGF1R* genes, which play important roles in the proliferation and apoptosis of hematopoietic cells [36–38]. However, our preliminary experiments show that the expression of these genes is not significantly repressed by *WT1* in M1 cells (data not shown). Also, although WT1 is reported to induce apoptosis in osteosarcoma cell lines by repressing transcription of the EGF receptor [15], M1 cells do not express the EGF receptor. In addition, wild-type p53 is not expressed in M1 cells. Thus, WT1-induced apoptosis observed in this study is independent of the EGF receptor and p53. The target genes important for the WT1-mediated induction of G1 arrest and apoptosis remain to be elucidated.

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