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Wilms' Tumor Gene Product WT1 Arrests Macrophage Differentiation of HL-60 Cells Through Its Zinc-Finger Domain

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Wilms' tumor is associated with mutations of WT1, a zinc-finger transcription factor that is essential for the development of the metanephric kidney and the urogenital system. High levels of WT1 expression also have been detected in myeloid leukemia cells, suggesting that WT1 may be important in other neoplasms as well. To seek a role of high level expression of WT1 in the differential arrest characteristic of myeloid leukemia, WT1 or its zinc-finger domain alone was stably expressed in human promyeloid leukemia (HL-60) cells and the ability of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to induce macrophage differentiation was examined. HL-60 cell differentiation was completely arrested in TPA treated cells that expressed WT1 or its zinc-finger domain alone whereas TPA fully induced macrophage differentiation in control HL-60 cells, indicating that high level expression of WT1 is capable of differentiation arrest of myeloid cells and that its effect may be mediated through its zinc-finger domain. To determine if the zinc-finger domain of WT1 directly influences transcription, it was brought to promoter DNA as a fusion protein with the Gal4 DNA binding domain. The fusion protein failed to regulate transcription of a reporter gene but when the zinc-finger domain of WT1 was brought to DNA with a promoter containing two upstream WT1-binding sites, reporter gene expression was activated approximately threefold, suggesting that WT1 interferes with myeloid differentiation through the ability of its zinc-finger domain to compete with other transcription factors for common promoter elements.

The Wilms' tumor susceptibility gene, wt1, encodes a C_2-H_2 type zinc- finger protein, WT1 (1, 2), that is implicated in both familial and sporadic Wilms' tumor (3). However, high levels of WT1 expression are found in human leukemia cells (8–12) and an inverse relationship between the state of differentiation of both normal and leukemia cells and levels of WT1 expression has been established (13, 14). WT1 mRNA is expressed in developing spleen and bone marrow (4–7). Furthermore, mutations in the zinc-finger encoding region of the WT1 gene are found in about 15% of acute myeloid leukemias (AML) (15,16) and WT1 antisence oligomers inhibit the growth and induce apoptosis of leukemia cells (17,18). The evidence strongly supports a role of WT1 in the development of leukemia.

To seek additional evidence for roles of WT1 in acute leukemia and the differentiation arrest that characterizes the leukemia cells, we used the HL-60 cell line (19) to determine whether constitutive expression of WT1 influences myeloid differentiation. HL-60 cells treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) differentiate into macrophage-like cells characterized by a loss of the ability to divide, the acquisition of adherence to surfaces of culture dishes, and appearance of monocyte/macrophage surface markers (19), providing an optimal model system to determine whether WT1 influences myeloid differentiation.

In these experiments, HL-60 cell lines were established that constitutively express WT1 or its zinc-finger domain alone. Surprisingly, the constitutive expression of either WT1 or its zinc-finger domain alone arrested TPA induced macrophage differentiation in HL-60 cells. The experiments also demonstrated that the zinc-finger domain of WT1 lacked intrinsic transcriptional regulatory activity but it stimulated expression of a reporter gene fused with a promoter that contains multiple WT1 binding sites about 2-3 fold, suggesting that the constitutive expression of WT1 may contribute to the differentiation arrest through its ability to compete with other

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transcription factors for binding to similar promoter recognition elements.

MATERIALS AND METHODS

Cell culture and DNA transfections. HL-60 cells were maintained in suspension culture in RPMI 1640 media supplemented with 10% FCS plus 1% penicillin and streptomycin (GIBCO). 1×10^7 of HL-60 cells were transfected by electroporation with 50 μg of linealized plasmid DNA. After recovery for 48 hours, the transfected cells were plated in 12-well dishes and selected with 500 μg/ml genectin (G418) for three weeks. Drug-resistant colonies were pooled and used for the experiments described below.

Expression vectors that contain the full-length cDNA of the WT1 alternatively spliced isoform that lacks the two inserts (-KTS and -17AA) (CMV-WT1) and its zinc-finger domain alone (CMV-WTZF) were constructed as described previously (20).

HL-60 cells were treated with 10 nM TPA for three days to induce macrophage differentiation. Morphological differentiation was established by analyzing 300 cells in cytopsin smears stained with May-Grunwald-Giemsa that were scored as immature blast cells, intermediate stage differentiation cells, and mature myeloid cells.

Western blot analysis. Cells were washed with PBS, lysed with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 1 mM PMSF and 10 μg/ml each of aprotinin and leupeptin. Cell lysates were boiled for 5 minutes and applied to 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose filters (BA85, S&S) and the filters were probed with a WT1 polyclonal antibody (C-19, Santa Cruz Biotechnology) which recognizes amino acids 431-449 of the zinc-finger domain of WT1, and visualized with an alkaline phosphatase-conjugated secondary antibody.

Plasmid construction and CAT assay. The chloramphinical acyltransferase (CAT) reporter plasmid p1X(GC)A- CAT was constructed as previously described (21). It has three contiguous WT1 binding sites fused upstream of the minimal promoter region of PDGF A-chain gene (-60 to $+3$, relative to the transcription start site). Another reporter plasmid p2X(GC)A-CAT was constructed by cloning an oligonucleotide containing three contiguous WT1 binding sites upstream of the original WT1 binding sites in the p1X(GC)A-CAT plasmid. For Gal4 fusion protein experiments, the 5XUASpSV-CAT reporter plasmid used was described before (21). The expression vector for the Gal4/ZF chimeric protein (pSG-ZF) was constructed by fusing the DNA fragment encoding amino acids 294-449 of WT1 in frame into the expression plasmid pSG424 that contains the SV40 early promoter that drives expression of Gal4 DNA binding domain (amino acids residues 1-147).

HL-60 cells (1×10^7) were transfected by electroporation using the reporter plasmid (5 μg), the expression plasmids in the amounts indicated in the figure legends, and a β-galactosidase expression plasmid (pCMVβ, Clontech) (1 μg) to establish transfection efficiency. The total amount of expression vector added in each transfection was adjusted to 20 μg by addition of the empty vector alone. Forty-eight hours after transfection, cell extracts were prepared, aliquots were normalized for transfection efficiency by assay of β-galactosidase activity, and CAT activity was determined. After autoradiographic exposure, the thin layer chromatographic (TLC) plates were assayed by scintillation counting and the percent conversion calculated.

RESULTS

WT1 mRNA levels fall sharply in TPA treated human promyelocytic HL-60 cells as they differentiate into macrophages (13), suggesting that down-regulation of WT1 gene expression is differentiation-linked and that WT1 may be important in establishing early macrophage differentiation. Because WT1 expression levels are high in many poorly differentiated human leukemias, we constitutively expressed the full-length human WT1 and separately, its zinc-finger domain alone and an empty expression vector (control) as described above. After selection with G418 (500 μg/ml) for three weeks, surviving cells were pooled, expanded and maintained in medium containing 200 μg/ml of G418. Western blots probed with a rabbit polyclonal anti-human WT1 antibody (Santa Cruz Biotechnology, CA) confirmed that a ~50 KD full-length WT1 protein and a ~27 KD protein containing the zinc-finger domain of WT1 were highly expressed in the transfected HL-60 cells (HL60-WT1 and HL60-WTZF) (Fig. 1).

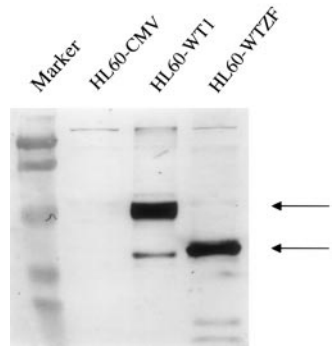

FIG. 1. Establishment of HL-60 cells expressing the full-length WT1 or zinc-finger domain of WT1. Western blot analysis of the WT1 expression in the control HL-60 cells transfected with empty expression vector (HL60-CMV), HL-60 cells transfected with the expression vector containing the full-length WT1, (HL60-WT1), and the zinc-finger domain of WT1 (HL60-WTZF).

To determine the influence of WT1 and its zinc-finger domain on macrophage differentiation pathway of HL-60 cells, the HL-60 cells were treated with TPA. After 3 days of treatment, the control HL-60 cells (HL60-CMV) were intensely adherent to the culture dish surface and to each other whereas HL-60 cells expressing WT1 (HL60-WT1) and the zinc-finger domain of WT1 (HL60-WTZF) grew continuously without evident differentiation (Table 1), suggesting that constitutive expression of both intact WT1 and its DNA binding (zinc-finger) domain have regulatory activity at the level of transcription in macrophage differentiation.

To determine if the zinc-finger domain of WT1 alone regulates transcription, it was brought directly to promoter by fusing it with a heterologous DNA-binding domain from the Gal4 transcription factor. The fusion protein was tested in co-transfection assays with a reporter plasmid containing five contiguous Gal4 binding sequences upstream of a minimal SV40 promoter (5XUASpSVCAT, 21). The zinc-finger domain of WT1

TABLE 1

Analysis of HL-60 Cell Differentiation in the Absence or Presence of Exogenous WT1 and the Zinc-Finger Domain of WT1

Cells	Inducer	Cell number (X 10^6 /ml)	Cell type (%) Mature	Intermediate	Blast
HL60-CMV	None	4.05	0.0	1.0	99
	TPA	0.33	75	25	0.0
HL60-WT1	None	2.05	0.0	1.0	99
	TPA	1.2	0.0	8.0	92
HL60-WTZF	None	2.2	0.0	2.0	98
	TPA	1.4	0.0	10.0	90

Note. The control HL-60 cells (HL60-CMV), the HL60-WT1 and the HL60-WTZF cells (4×10^5 cells per ml) were uninduced or induced to differentiate with 10 nM TPA for 3 days. Cells were counted using a hemocytometer, and cell types were determined from Wright-stained samples that included at least 300 cells. Immature blast cells were characterized by scant cytoplasm and round or oval nuclei; cells at the intermediate monocyte stage of differentiation were flattened, with a larger cytoplasm to nucleus ratio, and contained irregularly shaped nuclei and few or no interspersed vacuoles; mature macrophage-like cells were flattened and spread out, with numerous vacuoles interspersed in a greatly enlarged cytoplasm. All values represent the means of at least three independent experiments.

had no effect on the promoter activity of the reporter plasmid (Fig. 2), indicating that the zinc-finger domain of WT1 lacks intrinsic transcription regulatory activity and suggesting that its activity and that of WT1 itself in differentiation of HL-60 cells is mediated directly through its ability to bind to WT1 consensus sequences in promoter DNA.

To test this suggestion, the zinc-finger domain of WT1 was brought to promoter DNA through its ability to bind directly to WT1 binding sequences (Fig. 3). HL-60 cells were co-transfected with an expression vector that contains a cDNA encoding the zinc-finger domain of WT1 and a vector to encode CAT as a reporter driven by a minimal platelet-derived growth factor a-chain promoter (−60 to +3) containing one or two clusters of upstream WT1 binding sequences (p1X(GC)A-CAT or p2X(GC)A-CAT). When CMV-WTZF was co-transfected with the p1X(GC)A-CAT reporter plasmid, no change of promoter activity was observed; however, when p2X(GC)A-CAT was used with CMV-WTZF, promoter activity was activated between 2 to 3 fold. These data indicate that the zinc-finger DNA binding-domain of WT1 alone is capable of activating transcription of the reporter gene only when two or more molecules of the zinc-finger domain of WT1 bind to consensus WT1 binding sites in promoter region. The results suggest that the zinc-finger domain and perhaps WT1 itself arrest differentiation of promyelocytic HL-60 cells through its ability to displace transcription factors that themselves regulate transcription from common DNA recognition sites in promoter DNA.

DISCUSSION

In this manuscript, we establish that WT1 and its zinc-finger alone have the ability to arrest TPA-induced differentiation of HL-60 cells into the macrophages. This finding may be of general significance since WT1 also partially blocks the differentiation program of the human leukemia cell line U937 (22) and blocks the differentiation-inducing signal of G-CSF in the hematopoietic cell line 32D cl3 (23), suggesting that WT1 contributes to normal hematopoietic cell differentiation and that constitutive WT1 expression may contribute to the early differentiation arrest characteristic of human leukemias. The findings in these experiments also suggest a mechanism by which WT1 may contribute to differentiation arrest and thus to oncogenic transformation of hematopoietic progenitor cells. It was found that the zinc-finger domain alone induced differentiation arrest of HL-60 cells, suggesting that the zinc-finger domain of WT1 mediates the differentiation arrest associated with constitutive expression of WT1. Furthermore, the zinc-finger domain of WT1 activated promoter activity only when it was brought to promoter DNA via WTI specific recognition sequences but not when brought directly to DNA via a heterologous DNA binding domain, indicating that the zinc-

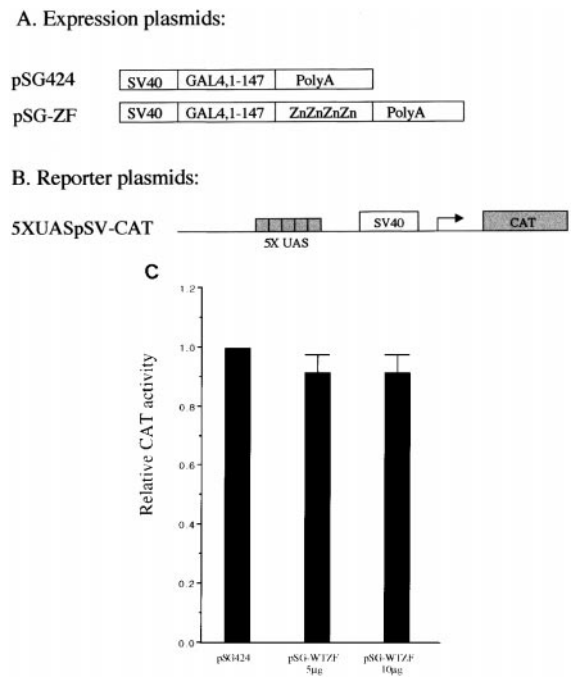

FIG. 2. Gal4/ZF fusion protein exhibits no intrinsic regulatory activity. (A, B) Schematic representation of the expression and reporter plasmids used in experiments. (C) CAT activities in lysates from the transfected cells were determined after co-transfecting into cells with pSG-ZF (5 and 10 μg) and 5XUASpSV-CAT (5 μg). The relative CAT activities are calculated and shown with standard deviation.

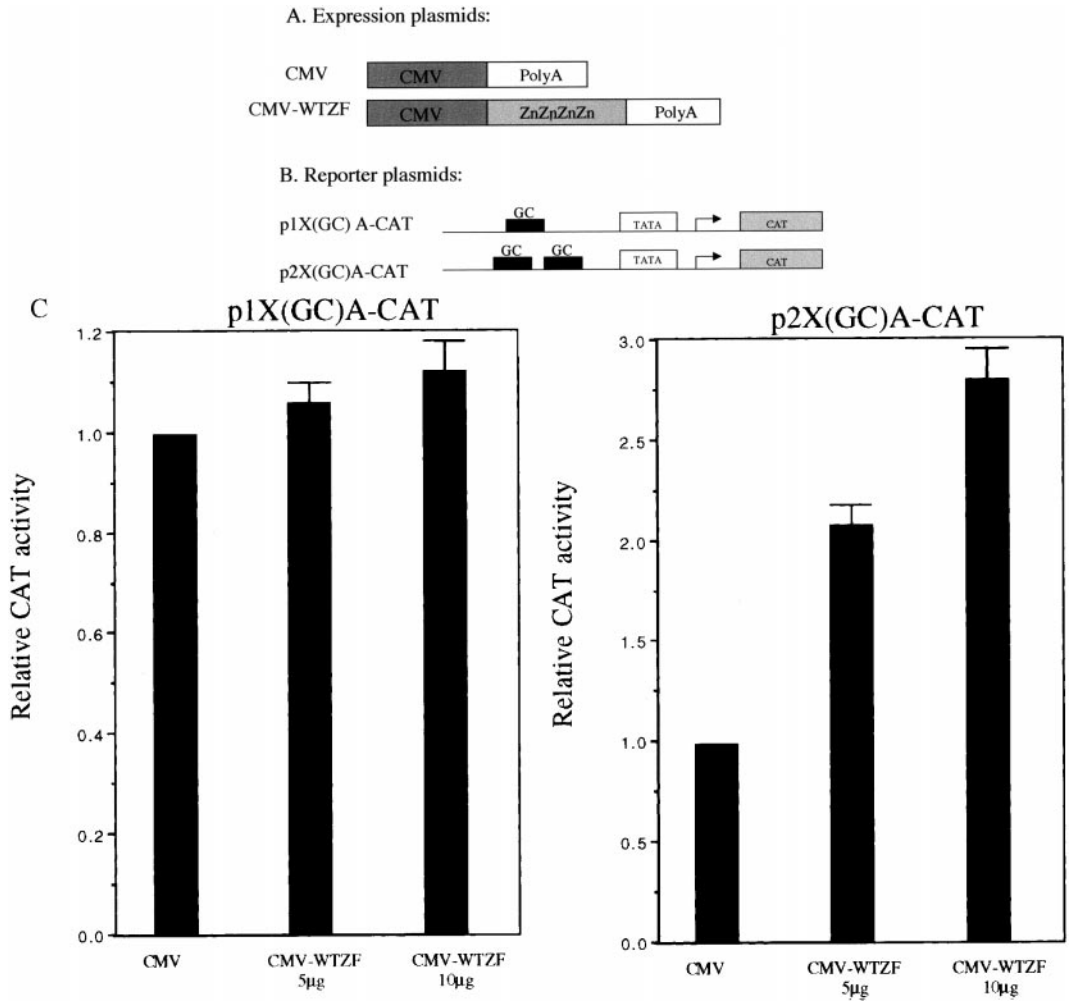

FIG. 3. Transcriptional activation by the zinc-finger domain of WT1. (A, B) Expression and reporter plasmids used for co-transfection assay. (C) CMV–WTZF expression vector at the amount indicated at the bottom of each column was co-transfected with reporter plasmids p1X(GC)A-CAT (5 μg), and p2X(GC) A-CAT (5 μg), into HL-60 cells. CAT activities in lysates from the transfected cells were analyzed, and the relative activities are calculated and shown with standard deviation.

finger domain alone has no intrinsic transcription regulation activity but raising the possibility that the zinc-finger domain of WT1 competes with other transcription factors important in differentiation of the macrophage lineage that recognize similar DNA-binding sequences. One such transcription factor may be the zinc-finger transcription factor Egr-1 or other members of the Egr-1 family. Egr-1 is a primary response gene that is essential for macrophage differentiation (24) and potentiates macrophage differentiation in the hematopoietic precursor cell line 32D cl3(25). Egr-1 and WT1 share strikingly structural similarity in their zinc-finger domains (2,3) and both recognize identical DNA binding sequences in promoter DNA. Interestingly, the zinc-finger DNA-binding domain of Egr-1 alone negatively regulates tumor cell growth (29), and, as noted above, point mutations have been detected in the zinc-finger domain of WT1 in acute myeloid leukemia (16,17), raising the possibility that mutations in the zinc-finger domain of transcription factors may disrupt differentiation of hematopoietic progenitor cells and lead to leukemia.

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