Growth Inhibition of Human Leukemic Cells by WT1 (Wilms Tumor Gene) Antisense Oligodeoxynucleotides: Implications for the Involvement of WT1 in Leukemogenesis

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We have previously reported expression of WT1 in acute leukemia. To elucidate its biological significance, we examined the effect of the suppression of the WT1 expression by WT1 antisense oligomers on the growth of the leukemic cells expressing WT1. When 20 different WT1 antisense (AS) oligomers covering from the 5' cap sites of the WT1 gene to the 3' end were examined for the inhibitory effect on the growth of K562 cells expressing WT1, four WT1 AS oligomers inhibited the cell growth, whereas WT1 sense and random sequence oligomers had no effect on the cell growth of K562. Moreover, WT1 AS oligomers significantly inhibited the growth of the clonogenic cells of fresh leukemic cells in six of 14 patients with acute myeloid leukemia, in one of two patients with chronic myelogenous leukemia (CML) chronic phase, and in one of one patient with CML blastic crisis. However, these oligomers did not inhibit normal colony-forming unit-granulocyte-macrophage. Western blot analysis clearly demonstrated the significant reduction in the WT1 protein levels in the K562 and fresh leukemic cells that were treated with the WT1 AS oligomers, confirming that the inhibitory effect of the WT1 AS oligomers on the cell growth operates via the reduction in the WT1 protein levels. These results show that WT1 plays an important role in leukemogenesis.

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MATERIALS AND METHODS

Patients. At the onset of the disease, bone marrow (BM) cells were obtained from leukemia patients before therapy. The leukemic cells were classified according to the French-American-British criteria. Normal BM cells were obtained from healthy volunteers with informed consent.

Purification of leukemic cells. Heparinized BM cells were suspen- ded in RPMI 1640 medium, put on Ficoll-Isoaque solution, and centrifuged. The leukemic cells were recovered from the interface, washed twice with RPMI 1640 medium, incubated in RPMI 1640 medium containing 10% fetal bovine serum (FBS) overnight to deplete adherent cells, and used for experiments. Microscopic examination showed the purity of the leukemic cells to be more than 90% in all cases.

Cell lines. The human hematopoietic cell lines, K562 (CML erythroid blast crisis), KG-1 (acute myeloid leukemia), THP-1 (acute monocytic leukemia), HEL and TF-1 (erythroleukemia), and U937 (histiocytic lymphoma) were kindly provided by the Japanese Cancer Research Resources Bank (Tokyo). DL-40 (non-Hodgkin lymphoma, diffuse large cell) was kindly provided by Dr. I. Kubonishi (Kochi Medical School, Kochi, Japan).

Oligodeoxynucleotides. Unmodified oligodeoxynucleotides were synthesized by using an automated synthesizer (Applied Biosystems, Foster City, CA), according to the published protocol. The oligomers were purified by high performance liquid chromatography (HPLC) and precipitated with ethanol three times, then resuspended in phosphate-buffered saline (PBS) for use.

The sense (SE) and antisense (AS) WT1 sequences are as follows: SE1 (transcription cap 1 site), 5'-CCCAACGCATTGCCACCT-3'; AS1, 5'-AGGGTGCAATGCCCTTGCGGG-3'; SE2 (transcription cap 3 site), 5'-CCGGCCCTCCTATTGGA-3'; AS2, 5'-TCAATAAGGAGGCCGCCGG-3'; SE3 (translation initiation site), 5'-CAGCAATATGGGTCTTCGACG-3'; AS3, 5'-TGCGGACGTGTGCTGTGCT-3'; SE4 (exon 6), 5'-AGCGATAACACACACAAAGC-3'; AS4, 5'-CGTGTGTTGTATTC-3'; myeloperoxidase AS oligomers, 5'-
AGAGAAGAAGGGAAACCCC-3'; coagulation factor V AS oligomers, 5'-GGCGTGCCAGCCTGGGAA-3'. Random sequences used as a control were 18-mer oligodeoxynucleotides, each deoxynucleotide of which was randomly synthesized. Thus, the random sequences theoretically are a mixture of 4^18 different 18-mer oligodeoxynucleotides.

Oligomer treatment of cells. The indicated numbers of cells were plated in RPMI 1640 medium without FBS. The oligodeoxynucleotides were added to the culture medium in triplicate at the indicated concentration for each experiment, and 2 hours later, FBS was added to the culture medium at a final concentration of 10% (afterward the culture medium contained 10% FBS throughout culture). The same oligodeoxynucleotides were added to each well to a final concentration equivalent to one-second of the initial concentration every 24 hours for the indicated days for each experiment. The rate of cell growth was determined by counting the viable cells using dye neutral red (DNR) assay. Open and closed columns represent W1 sense and antisense oligomers. The WTI oligomer concentration was determined by measuring the number of colonies formed after 4 days of culture. Arithmetical means, standard errors, and statistical significance of differences between means of test groups were assessed by Student's t-test.

RESULTS

Inhibition of leukemic cell growth by WT1 antisense oligomers. We examined the effect of WT1 AS oligomers on the growth of K562 cells, which express WTI. The random, WT1 SE and AS oligomers were added to the culture medium of K562 cells at a concentration of 200 μg/mL (10^-12 to 23 μmol/L). As shown in Figs 1 and 2, four (AS1, AS2, AS3, and AS4) of 20 different AS oligomers covering from the 5' cap sites of the WT1 gene to the 3' end significantly inhibited the cell growth as compared with the random and WT1 SE oligomers. The WT1 SE2 oligomer also inhibited the growth of K562 cells by WT1 antisense oligomers. The oligomers were added to the culture medium of K562 cells (5 x 10^4 cells/well in 96-well plate) at a concentration of 200 μg/mL, and then three times at a final concentration of 100 μg/mL every 24 hours. The viable cells were counted 4 days after the initial treatment with the oligomers. Open and closed columns represent WT1 sense and antisense oligomers, respectively. **P < .01; ***P < .001.
the treatment with the WT1 oligomers for the indicated days. K562 cells were plated at 2.5 x 10^4 cells/well in 24-well plate. The WT1 sense (SE3) and antisense (AS3) oligomers were added to the culture medium at a concentration of 200 µg/mL and then at a concentration of 100 µg/mL every 24 hours for the indicated days. The viable cells were counted after 4 days of treatment with the WT1 oligomers for the indicated days. (C), WT1 sense oligomer (SE3); ( ), WT1 antisense oligomer (AS3); ( ), control without oligomers.

The growth inhibition might be due to the antisense effect of the WT1 sense oligomers on the transcripts of the Wit-1 gene, which is transcribed in an antisense direction (for details, see Discussion). Because the WT1 AS3 oligomer had an antisense effect stronger than other WT1 AS oligomers, the WT1 AS3 oligomer was mainly used for the further experiments.

When the WT1 AS3 oligomer was added to the culture medium of K562 cells at varying concentrations, the WT1 AS3 oligomer concentration-dependently inhibited the cell growth, whereas the WT1 SE3 oligomer did not significantly inhibit the cell growth even at a higher concentration (320 µg/mL) (Fig 3). The WT1 AS4 oligomer also concentration-dependently inhibited the cell growth of K562, whereas the WT1 SE4 oligomer did not have any effect on the cell growth even at a higher concentration (data not shown). Time course of the cell growth of K562 that was treated with the WT1 AS3 oligomer was measured (Fig 4). The WT1 AS3 oligomer significantly inhibited the cell growth, whereas the WT1 SE3 oligomer had no significant effect on the cell growth.

Specific inhibition of leukemic cell growth by WT1 antisense oligomers. When the WT1 SE3 and AS3 oligomers, myeloperoxidase AS or coagulation factor V AS oligomers were added to the culture medium of K562 cells, only the WT1 AS3 oligomer significantly inhibited the cell growth, indicating that the antisense effect observed here was not due to a toxic effect of the oligomers (Fig 5). Moreover, when the WT1 AS oligomers were added to the culture medium of the cell lines expressing or not expressing the WT1 gene, the cell growth was inhibited in both of two cell lines expressing the WT1 gene (HEL and THP-1, Figs 6A and B, respectively), but not in the cell line not expressing the WT1 gene (U937, Fig 6C). These results showed that the inhibitory effect of the WT1 AS oligomers on the leukemic cell growth was specific.

Growth inhibition of the fresh leukemic cells isolated from leukemia patients by WT1 antisense oligomers. When the leukemic cells that were freshly isolated from leukemia patients were treated with the WT1 AS3 oligomer, the colony formation of the leukemic cells was significantly inhibited in eight of 17 patients with acute or chronic myeloid leukemia (Table 1 and Fig 7). The antisense effect was observed regardless of the subtypes of AML or stages of CML, although it appeared to be stronger in CML blastic crisis than in CML chronic phase. The representative cases are shown in Fig 7. The colony formation of the leukemic cells from a patient with CML blastic crisis was increased by the treatment with the WT1 SE3 oligomer, as compared with control without oligomers. However, this increase was statistically not significant.

No effects of WT1 antisense oligomers on normal CFU-GM. We determined the effects of the WT1 AS oligomers on normal hematopoietic cells. The WT1 AS oligomers were added to the culture medium of BM cells freshly isolated from four healthy volunteers, and the cells were assayed for CFU-GM (Fig 8). No inhibition of CFU-GM was observed in BM cells of all four healthy volunteers.

Reduction of the WT1 protein levels by WT1 antisense oligomers. To confirm the specificity of the anti-WT1 antibodies used here, cell lysates from various kinds of cell lines were subjected to Western blot analysis (Fig 9A). WT1-expressing KG-1, HEL, and TF-1 cell lines produced two bands of 54 and 52 kD, while full-sized WT1 cDNA-transfected U937 cells produced only one band of 54 kD. These results confirmed the specificity of the anti-WT1 antibodies.

When added to the culture medium of K562 cells, the WT1 oligomers produced a significant decrease in the WT1 protein levels (Fig 9B). In contrast, the control random oligo-
LEUKEMIC CELL GROWTH AND WT1 ANTISENSE ODN

Fig 6. Growth inhibitory effects of WT1 antisense oligomers dependent on the WT1 expression in cells. The random and WT1 SE and AS oligomers were added to the culture medium of the HEL (A) and THP-1 cells (B) (5 x 10^5 cells/well in 96-well plate) expressing high levels of WT1, or of the U937 cells (C) not expressing WT1 at a concentration of 200 µg/mL, and then three times at a concentration of 100 µg/mL every 24 hours. The viable cells were counted 4 days after the initial treatment with the oligomers. Open and closed columns represent WT1 sense and antisense oligomers, respectively. **P < .01; ***P < .001.

Fig 7. Growth inhibition of the clonogenic leukemic cells by WT1 antisense oligomers. The WT1 sense (SE3) and antisense (AS3) oligomers were added to the culture medium of the fresh leukemic cells (1.5 x 10^5 cells/well in 96-well plate) from the patients with acute or chronic myelogenous leukemia at a concentration of 200 µg/mL, and then three times at a concentration of 100 µg/mL every 24 hours. Four days after the initial treatment with the WT1 oligomers, the leukemic cells were harvested, seeded (5 x 10^3 cells/dish) and cultured in methyl cellulose-containing medium as described in Materials and Methods. Open and closed columns represent WT1 sense and antisense oligomers, respectively. *P < .05; **P < .01; ***P < .001.

**DISCUSSION**

In this study, we clearly demonstrated that the suppression of WT1 expression by WT1 AS oligomers inhibited the growth of leukemic cells, implying the involvement of WT1 in leukemogenesis. The most important point to note in the experiments using antisense oligomers is whether the phenomenon observed is specific to the inhibition of targeted genes. Antisense effect of the WT1 AS oligomers observed here is specific to the inhibition of the WT1 gene expression. Our present results are specific to the inhibition of the expression of the WT1 gene for the following reasons: (1) only four AS oligomers of WT1 sense and antisense oligomers (a total of 40 different WT1 oligomers) of 20 different regions were treated with WT1 AS oligomers at a concentration of 200 µg/mL for 4 days. Percent growth to random oligomer-treated control for nonspecific effects of oligomers are shown in Fig 10. These results showed that the transfection of a CMV-driven WT1 cDNA restored the antisense effects, although it was not complete, indicating that the inhibition of leukemic cell growth by WT1 AS oligomers resulted from the suppression of WT1 gene expression. One explanation for incomplete restoration of the CMV-driven WT1 cDNA-transfected K562 cells might be that since WT1 AS oligomers could have antisense effects on at least four different WT1 transcripts (one nonspliced and three spliced), transfection of a full-sized (nonspliced type) WT1 cDNA alone could not completely restore the antisense effects.

**Table 1. Inhibition of Colony Formation of Fresh Leukemic Cells by WT1 AS Oligomers**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No. of Patients Examined</th>
<th>No. of Patients for Whom AS Oligomers Were Effective</th>
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<tbody>
<tr>
<td>AML</td>
<td>M1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>4</td>
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<td>2</td>
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<tr>
<td></td>
<td>BC</td>
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</tr>
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Total 17 8
covering from the cap sites of the WT1 gene to the 3' non-coding region exhibited the antisense effect on the inhibition of leukemic cell growth (Fig 1); (2) antisense effect of WT1 AS oligomer was exhibited only on the cell lines expressing WT1, but not on the cell line not expressing WT1; (3) most importantly, the growth inhibition of leukemic cells by the WT1 AS oligomers was associated with the reduction in the WT1 protein levels in these cells. The experiments showing the reduction in WT1 protein levels were performed at a concentration of 200 μg/mL of the WT1 AS oligomers. As this concentration is equivalent to 20 to 23 μmol/L, it is not so high and is reasonable.14-17 The WT1 protein levels in the AS oligomers-treated K562 cells were measured at two time points (days 3 and 4) after the start of the treatment. At both time points, significant reduction in the WT1 protein levels was observed; and (4) constitutive expression of the WT1 gene by the transfection of a CMV-driven WT1 cDNA restored the antisense effects of WT1 AS oligomers.

The growth inhibition of K562 cells by the WT1 sense oligomers, SE2, was observed (Fig 2). Sequences upstream from WT1 have been cloned, including the Wit-1 gene, which is approximately 2 kb upstream.14-23 WT1 and Wit-1 are transcribed in the same temporal and cell-restricted pattern, although Wit-1 expression is less abundant than WT1 expression.22 However, the role of Wit-1 remains unclear. WT1 and Wit-1 were thought to be bidirectionally transcribed from the same promoter region.19 Campbell et al25 have recently reported multiple transcriptional start sites of Wit-1, and bidirectional transcription within a region of DNA that included exon 1 of WT1. Moreover, Eccles et al21 have recently detected a novel 7 to 10-kb transcript in human fetal kidney, which contains part of intron 1 of WT1, exon 1, upstream sequences between WT1 and Wit-1 and part of the Wit-1 gene. Because this transcript is detected by Northern blot analysis using the DNA probe containing the sequences ranging from exon 2 of WT1 to exon 5, Wit-1 transcription may start within at least intron 2 of WT1 and proceed in an antisense direction. Thus, the WT1 sense oligomers, SE2, for the promoter region of the WT1 gene (intervening sequence between the Wit-1 and WT1 genes) might have antisense effects on the Wit-1 gene function, although it is unknown, resulting in the growth inhibition of K562 cells.

Fig 8. No effects of WT1 antisense oligomers on normal CFU-GM. The WT1 sense (SE3) and antisense (AS3) oligomers were added to the culture medium of the normal bone marrow cells from healthy volunteers at a concentration of 200 μg/mL, and then CFU-GM was assayed through the same processes as those in Fig 7. Open and closed columns represent WT1 sense and antisense oligomers, respectively.
WT1 AS oligomers inhibited the growth of the fresh leukemic cells in six (43%) of 14 patients with AML and two (67%) of three patients with CML (Table 1). There was no significant correlation between the WT1 expression levels in the leukemic samples and the effectiveness of the WT1 AS oligomers on the growth inhibition of the leukemic cells (Table 2). As shown in Table 2, we could not observe an obvious correlation between the WT1 expression levels and the degree of growth inhibition. However, this does not necessarily mean no correlation between these two matters. This discrepancy might be because the WT1 expression levels measured here reflect the whole leukemic cells in the samples that consist of heterogenous leukemic cells with varying levels of WT1 expression and do not necessarily reflect the WT1 expression levels of clonogenic cells (immature clonogenic cells express WT1 more strongly than more mature, differentiated cells as described previously), and because the impairment of the WT1 function by the WT1 AS oligomers could be compensated by the other genes, the expression levels of which varied from case to case.

The antisense oligomers for proto-oncogenes, such as c-myb, B-myb, and c-myc, and for oncogenic fused genes, such as bcr-abl and AML-ETO genes were examined for the inhibitory effects on the leukemic cell growth. All of these antisense oligomers exhibited an inhibitory effect on the cell growth. The c-myb proto-oncogene is preferentially expressed in hematopoietic cells, and its encoded protein, Myb, is required for hematopoietic cell proliferation. Calabretta et al. demonstrated that c-myb antisense oligomers strongly inhibited or completely abolished clonogenic growth of a T-cell leukemia line, 78% (18 of 23) of acute myeloid leukemia cases examined, and four of five chronic myelogenous leukemia (CML) cases in blast crisis. At antisense doses that inhibited leukemic cell growth, normal hematopoietic progenitor cells survived. Moreover, Ratajczak et al. showed that c-myb antisense oligomers substantially inhibited the growth and survival of CML CFU, not only in blast crisis, but also in chronic phase. Thus, antisense effect of the WT1 antisense oligomers appears to be similar to those of c-myb antisense oligomers, in a sense that antisense effect of these two antisense oligomers is not restricted to types of leukemia, but found in various types of leukemia, including AML, CML chronic phase and blast crisis without damage to normal hematopoietic progenitor cells.

A potential functional interaction between WT1 and p53 was suggested by transactivation assays. While WT1 enhances transcriptional activation by p53, wild-type p53 appears to convert WT1 from a transcriptional activator to transcriptional repressor. These findings suggest that WT1 could interact with many other cellular proteins that regulate cell growth and differentiation. Therefore, it may be plausible that the dysexpression of wild-type WT1, although the mechanisms remain unknown, dysregulates the transcription of the target genes and interaction with cellular proteins that regulate cell growth and differentiation, resulting in the development of leukemia in cooperation with other oncogenes.

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