[CANCER RESEARCH 58, 3773–3776, September 1, 1998]

Advances in Brief

Antisense Oligodeoxyribonucleotide against the *MLL-LTG19* Chimeric Transcript Inhibits Cell Growth and Induces Apoptosis in Cells of an Infantile Leukemia Cell Line Carrying the t(11;19) Chromosomal Translocation[1]

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Abstract

To clarify the role of the multiple lineage leukemia gene-leukemia translocation gene of chromosome 19 (MLL-LTG19) protein in leukemogenesis, we synthesized antisense oligodeoxyribonucleotide (ODN) against the fused region of the *MLL-LTG19* chimeric transcript and treated KOCL33 cells carrying the t(11;19) translocation with antisense ODN. The antisense ODN inhibited cell growth and induced apoptosis in KOCL33 cells but not in Daudi cells, which have no t(11;19). The levels of *MLL-LTG19* mRNA and MLL-LTG19 protein in KOCL33 cells treated with antisense ODN were shown to decrease with time by reverse transcription-PCR and Western blot analysis. These results suggest that the MLL-LTG19 fusion protein contributes to cell proliferation and malignant transformation in infantile acute leukemia cells carrying the t(11;19) translocation.

Introduction

Chromosome band 11q23 is a common breakpoint of chromosome translocations in a variety of leukemias and lymphomas such as infantile leukemias and secondary leukemias associated with epipodophyllotoxin treatment (1, 2). Generally, leukemias carrying 11q23 translocations have a poor prognosis (1). Two groups cloned *MLL*,[3] which is involved in t(4;11)(q21;q23) (3) and t(11;19)(q23;p13) (4). We also cloned the breakpoint of t(11;19)(q23;p13) translocations observed in the KOCL33 and KOCL44 cell lines originated from infantile acute leukemias and found that the *MLL-LTG19/ENL* gene is formed by the t(11;19) translocation (5, 6). Furthermore, it was found that *MLL* is involved in t(11;17) and t(11;22) translocations (7–9), and it was concluded that *MLL* is responsible for the majority of 11q23 translocations. A sequencing study demonstrated that *MLL* is related to the *Drosophila trithorax* gene, encoding a protein containing two DNA-binding motifs as a transcriptional factor (3, 4). The molecular mechanism of leukemogenesis in 11q23 translocations involving *MLL* is explained by the fact that the chimeric proteins from *MLL*-partner genes are produced in a nonregulated manner, resulting in the dysfunction of MLL protein, which could be the cause of leukemogenesis (3, 4, 10).

Based on these findings, we sought to inactivate the chimeric transcript of *MLL-LTG19* observed in KOCL33 cells carrying the t(11;19) translocation by treating the cells with antisense ODN. As a result, we showed that it inhibited cell growth and induced apoptosis in KOCL33 cells.

Materials and Methods

Antisense ODNs. The 16-mer antisense phosphorothioate ODN complementary to the coding sequences of the *MLL-LTG19* (6) junction cDNA (antisense *MLL-LTG19*, 5′-ACGGTGCACTTAAAGT-3′) was synthesized and purified as described previously (11). As a negative control, we also prepared the sense phosphorothioate ODN corresponding to the same region as the antisense ODN (sense *MLL-LTG19*, 5′-ACTTTAAGTGCACCGT-3′).

Cell Culture and Treatment of KOCL33 Cells with Antisense or Sense ODN. KOCL33 is a human leukemia cell line derived from a patient with a B-cell infantile acute leukemia carrying the t(11;19)(q23;p13) translocation. Another human B-cell leukemia cell line, Daudi cells, which have no t(11;19)(q23;p13), was used as a control. Cells from these cell lines were seeded at a concentration of 5×10^5 cells/ml in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum 1 day before treatment. The treatment was performed by culturing these cells in serum-free medium with 1 μM antisense *MLL-LTG19* or sense *MLL-LTG19* complexed with 4 μM *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (12) in 60-mm diameter wells. After an 8-h exposure, we replaced the medium with conventional medium after centrifugation. For cell counting, cell cultures of KOCL33 or Daudi cells were started at 3×10^5 cells/ml in RPMI 1640 containing 10% fetal bovine serum, and the cell number was counted daily for 7 days. We designated the day of treatment as day 0.

Assessment of Cell Viability and Apoptosis. Cell viability was assessed by the ability of the cells to exclude trypan blue. The morphological characteristics of apoptosis were assessed by staining the cells with Hoechst 33342. At the same time, DNA was extracted from the cells and used to detect nucleosomal DNA fragmentation.

RT-PCR. Total RNA was extracted from KOCL33 and Daudi cells. The cells were washed three times with PBS, and total RNA was extracted by the phenol/guanidinium thiocyanate method followed by DNase I treatment (13). Using cDNA generated after the reverse transcription of 2 μg of total RNA, amplification of the chimeric *MLL-LTG19* cDNA region was conducted by PCR. PCR primers were as follows: *(a)* for *MLL*, P_1 (sense), 5′-CCTGCCCAAAGAAAAGCAG-3′ in exon 5; and *(b)* for *LTG19*, P_2 (antisense), 5′-GTTGAAGGTGAGCTTCTCGC-3′. These primers can specifically amplify the DNA fragment covering the fusion region of *MLL-LTG19* cDNA. The 661-bp β-actin cDNA was used as an internal standard. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, and 72°C for 1 min) after an initial denaturation step (95°C for 1 min). PCR products were analyzed by electrophoresis on 2% agarose gels.

Western Blot Analysis. KOCL33 cells with or without treatment were washed twice with PBS, suspended in lysis buffer (2× PBS, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride), and then homogenized with an ultrasonic homogenizer (Heat Systems-Ultrasonics, Farmingdale, NY). The homogenized samples were used without centrifugation. Lysate protein (80 μg) was separated by SDS-PAGE using a 6% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (DuPont New England Nuclear, Boston, MA). After blocking nonspecific binding sites for 1 h with 5% nonfat milk in PBS containing 0.1%

Received 4/17/98; accepted 7/10/98.

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[1] Supported in part by a grant from the Children's Cancer Association of Japan and a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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[3] The abbreviations used are: *MLL*, multiple lineage leukemia gene; *LTG19*, leukemia translocation gene of chromosome 19; ODN, oligodeoxyribonucleotide; RT-PCR, reverse transcription-PCR.

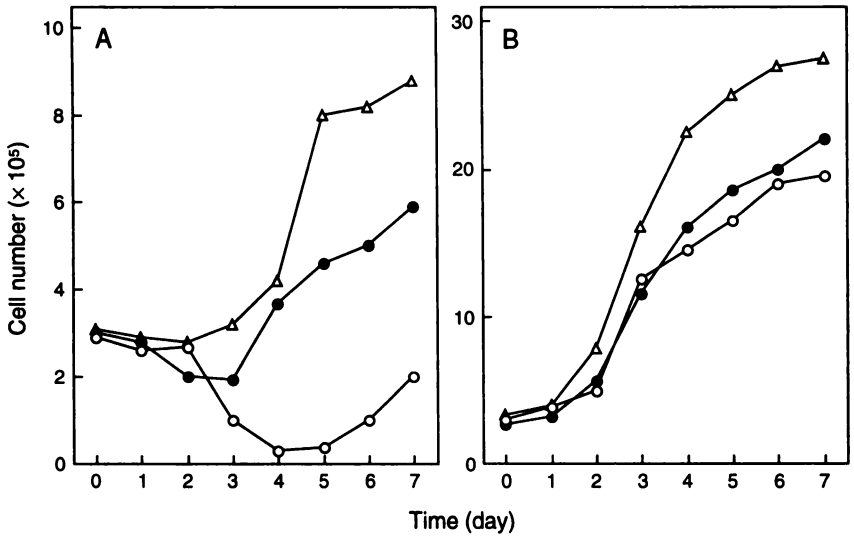

Fig. 1. Growth inhibition of KOCL33 and Daudi cells treated with antisense ODN against *MLL-LTG19* mRNA. *A*, KOCL33 cells inoculated at 5×10^5 cells/ml 1 day earlier were treated with 1 μM sense *MLL-LTG19* (●) or 1 μM antisense *MLL-LTG19* (○). △, cells without treatment. The starting cell number was set at 3×10^5 cells/ml. *B*, Daudi cells were treated in the same way as described above.

Tween 20, the membrane was incubated overnight at 4°C with antihuman MLL antibody (14) at a dilution of 1:60. The membrane was then washed three times with PBS containing 0.1% Tween 20, incubated with alkaline phosphatase-conjugated goat antirabbit antibody (Promega, Madison, WI) at room temperature, and then washed three times with PBS containing 0.1% Tween 20. The immunoblot was visualized using an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA).

Results

To evaluate the role of the MLL-LTG19 protein in the cell proliferation of KOCL33 cells with the t(11;19) translocation, we treated the cells with antisense *MLL-LTG19* or sense *MLL-LTG19*. Cell growth was gradually inhibited over a period of 4 days after the addition of antisense *MLL-LTG19* but was minimally affected by sense *MLL-LTG19*, as compared with the growth of the control cultures (Fig. 1*A*). The bottom of the growth curve appeared from day 4 to day 5; after that, the cell number gradually increased. In the case of Daudi cells, the cell growth after the addition of sense *MLL-LTG19* was also slightly inhibited. However, we found that the growth inhibition rate of Daudi cells treated with antisense *MLL-LTG19* was the same as that observed with sense *MLL-LTG19* (Fig. 1*B*).

The morphological aspects of KOCL33 cells at 4 days after the addition of antisense *MLL-LTG19* or sense *MLL-LTG19* were examined by Hoechst 33342 nuclear staining. Findings characteristic of apoptosis, such as cell shrinkage, chromatin condensation, and nuclear segmentation, were observed in KOCL33 cells treated with antisense *MLL-LTG19* (Fig. 2). Moreover, nucleosomal DNA fragmentation in such cells was shown by agarose gel electrophoresis (Fig. 3).

Using RT-PCR, we examined the level of *MLL-LTG19* mRNA in KOCL33 cells after treatment with antisense *MLL-LTG19* or sense *MLL-LTG19*. The amount of 824-bp PCR product from *MLL-LTG19* mRNA gradually decreased from day 3 to day 5 after the start of treatment with antisense *MLL-LTG19* in comparison with that obtained by treatment with sense *MLL-LTG19* (Fig. 4*A*). Although it is possible that the antisense ODN contaminating the cDNA sample of

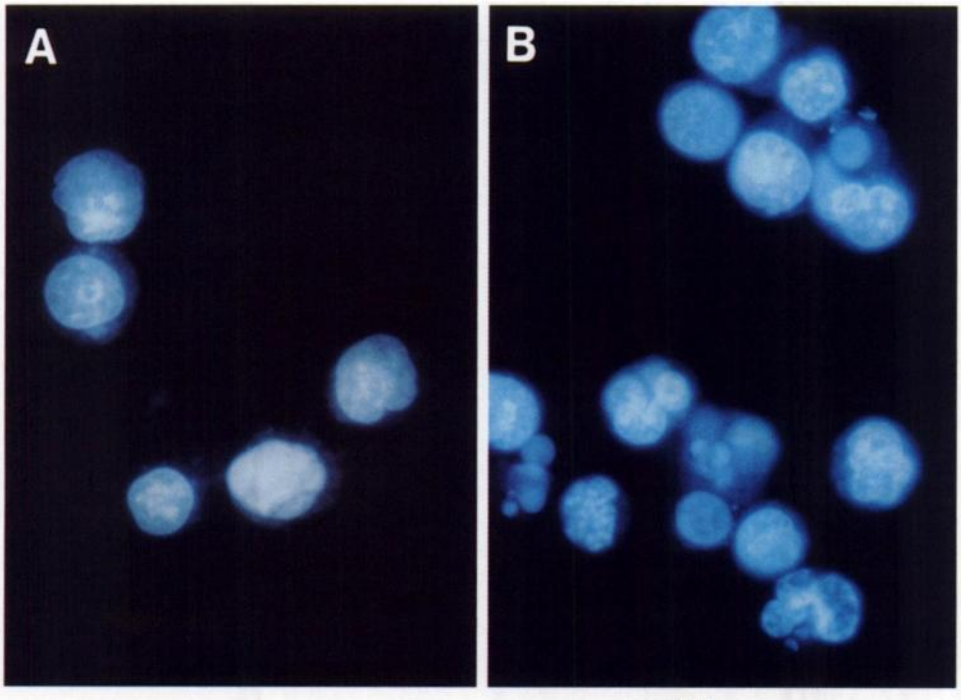

Fig. 2. Morphological aspects of cell death in KOCL33 cells treated with antisense *MLL-LTG19*. *A*, cells treated with sense *MLL-LTG19*; *B*, cells treated with antisense *MLL-LTG19*.

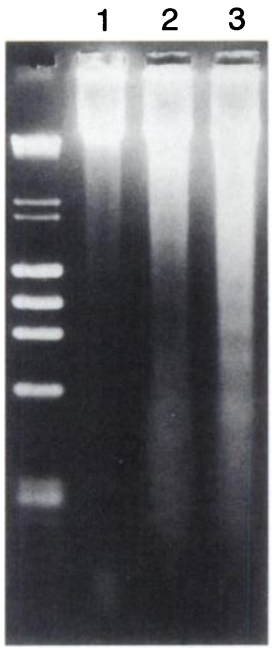

Fig. 3. Nucleosomal DNA fragmentation in KOCL33 cells treated with antisense *MLL-LTG19*. *Lane 1*, DNA from cells without treatment; *Lane 2*, DNA from cells treated with sense *MLL-LTG19*; *Lane 3*, DNA from cells treated with antisense *MLL-LTG19*.

KOCL33 cells treated with antisense *MLL-LTG19* inhibits the PCR, resulting in the reduction of the PCR product level, the difference in PCR products on day 3 and day 5 is derived from a reduction in the amount of *MLL-LTG19* mRNA. RT-PCR showed that the level of *MLL* mRNA was not changed by treating KOCL33 cells with antisense *MLL-LTG19* (data not shown).

We examined the change in the MLL-LTG19 protein of KOCL33 cells after treatment with antisense ODN by Western blot analysis. The amount of MLL-LTG19 protein was markedly decreased at day 5 after treatment with antisense *MLL-LTG19* in comparison with that with after treatment sense ODN (Fig. 4*B*).

Thus, the treatment of KOCL33 cells with 1 μM antisense *MLL-LTG19* caused cell growth inhibition and induced apoptosis by reducing the MLL-LTG19 chimeric protein level in KOCL33 cells.

Discussion

In the present study, we elucidated the role of the chimeric protein produced by chromosomal translocations in leukemogenesis and examined the possibility of treatment for leukemias carrying 11q23 translocations involving *MLL*. The infantile acute leukemias carrying 11q23 translocations are very resistant to chemotherapy and have a poor prognosis (1). Using antisense ODN targeted to the fused region of the *MLL-LTG19* chimeric transcript, we demonstrated that the antisense ODN substantially inhibited the growth of KOCL33 cells carrying t(11;19) and rendered these cells highly susceptible to apoptosis. The production of the MLL-LTG19 fusion protein was suppressed in parallel with the decrease in the *MLL-LTG19* chimeric transcript level after treatment. The down-regulation of MLL-LTG19 protein by antisense *MLL-LTG19* seems to be specific for KOCL33 cells, which have the t(11;19) translocation, because this antisense ODN was not effective in Daudi cells.

Vaerman *et al.* (15) reported a nonantisense cytotoxic effect of ODNs produced by exonuclease, which should be taken into account when performing any experiment using antisense ODN. We treated KOCL33 cells with ODNs in a serum-free condition and observed a difference in cell growth between sense ODN- and antisense ODN-treated cells. These findings suggest that the resultant MLL-LTG19 chimeric product elicited by the t(11;19) translocation is responsible for leukemogenesis.

It is well known that all-*trans*-retinoic acid induces patients with acute promyelocytic leukemia to complete remission (16). All-*trans*-retinoic acid leads acute promyelocytic leukemia cells carrying t(15;17) translocations to differentiate and induces apoptosis by the down-regulation of the promyelocytic leukemia retinoic acid receptor α chimeric protein. In addition, McGahon *et al.* (17) reported that antisense ODN targeting the *BCR-ABL* chimeric transcript down-regulated the expression of the BCR-ABL chimeric protein, which rendered chronic myelogenous leukemia K562 cells susceptible to apoptosis, although typical nucleosomal DNA fragmentation was not observed. Mitani *et al.* (18) demonstrated that antisense ODN against *AML1-EVI-1* inhibited the growth of leukemic cells carrying t(3;21) progressed from chronic myelogenous leukemia; although in this case also, no apoptic features were found.

These findings indicate that chimeric proteins such as promyelocytic leukemia retinoic acid receptor α and BCR-ABL, produced by chromosomal translocations, may be directly linked to leukemogenesis. The deregulation of these transcriptional factors could cause leukemias and lymphomas in a dominant negative manner. In our study, the cell death induced by antisense ODN against the *MLL-LTG19* transcript was typical apoptosis characterized by both nucleosomal DNA fragmentation and morphological aspects such as cell shrinkage, nuclear segmentation, and chromatin condensation. We suppose that the introduction of antisense ODN relieves KOCL33 cells from the dysfunction of the MLL protein due to its chimeric product and causes active gene expression for apoptosis by inhibiting MLL-LTG19 protein production.

Although expression vectors must enter the nuclei of transfected cells to undergo transcription, in the case of antisense ODN, it is expected that they can work efficiently to target mRNA in the cytoplasm, resulting in heightened therapeutic efficiency. With regard to the growth inhibition induced by antisense ODN, the cell growth suppression occurred slowly, over 3 days after the start of treatment. If antisense ODN treatment is not performed thereafter, the cell

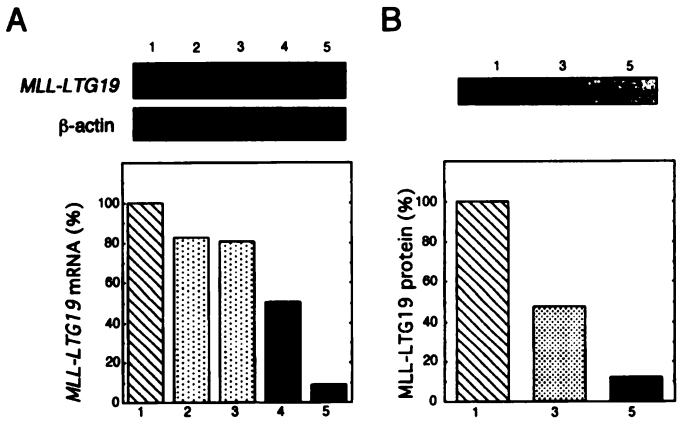

Fig. 4. Efficiency of antisense ODN against *MLL-LTG19* mRNA in KOCL33 cells as examined by RT-PCR and Western blot analyses. *A*, PCR was carried out to detect the *MLL-LTG19* chimeric transcript. The expected size of the product of *MLL-LTG19* mRNA with *MLL-LTG19*-specific primers is 824 bp. The internal control is 661-bp β-actin cDNA. *Lane 1*, KOCL33 cells without treatment; *Lanes 2* and *3*, KOCL33 cells treated with sense *MLL-LTG19* at day 3 and day 5, respectively; *Lanes 4* and *5*, KOCL33 cells treated with antisense *MLL-LTG19* ODN at day 3 and day 5, respectively. *B*, Western blot analysis of MLL-LTG19 protein with anti-MLL antibody. The molecular weight of MLL-LTG19 protein is 213,000. *Lanes 1*, *3*, and *5* correspond to those described in *A*. Quantification of the changes in mRNA and protein was performed by scanning the RT-PCR and Western blot film after analysis using an NIH Image System (*lower panels* in *A* and *B*).

number gradually recovered from day 5 after the start of treatment. This must be taken into account for the protocol of antisense ODN therapy.

Recently, it was reported that antisense ODN against *BCL-2* was effective on non-Hodgkin's lymphomas (19) and melanomas grown in laboratory animals (20). Although there are still many barriers to overcome, we consider that antisense ODN treatment twice a week in combination with chemotherapy and/or other cytoreduction therapies might be ideal for killing leukemia and lymphoma cells. For this purpose, the establishment of an antisense ODN delivery system will be essential.

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