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Inhibitory Effect of ATF3 Antisense Oligonucleotide on Ectopic Growth of HT29 Human Colon Cancer Cells

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ATF3 is a transcription factor belonging to the Jun/Fos family whose mouse homologue (TI-241) was isolated, using the differential screening method, from B16 mouse melanoma cells as a blood-borne metastasis-associated gene. Here we show the tumorigenicity-inhibiting effect of an antisense oligonucleotide designed to reduce the expression of ATF3 in HT29 colon cancer cells. HT29 cells were reported to metastasize to the skin after intravenous inoculation. The antisense oligonucleotide inhibited cell attachment to the collagen-coated floor of the plates and invasion of HT29 cells *in vitro*, which are thought to be two important factors in the process of cancer metastasis and ectopic tumor growth. While the antisense oligonucleotide had no effect on cell growth of HT29 cells *in vitro*, mice that were inoculated subcutaneously with HT29 cells and treated with the antisense oligonucleotide survived longer than the control mice due to the inhibition of tumor growth *in vivo*. These show that ATF3 plays an important role in the ectopic growth/metastasis of HT29 colon cancer cells.

Key words: ATF3—Phosphorothioate oligonucleotide—Inhibitory effect

In spite of progress in therapy, tumor metastasis is still the main cause of death in patients with cancer because of their resistance to immuno/chemotherapy. Tumor metastasis comprises sequential steps: growth at the primary site, vascularization, invasive attachment to capillary endothelial cells, and growth in target organs.[1, 2)] The critical genetic events that control the whole process are still unknown. To clarify this point we performed differential screening between mouse B16 melanoma sublines B16-F10 and B16-BL6. B16-F10 cells were established by 10 successive selections for lung metastasis following intravenous injection, and B16-BL6 cells were established from B16-F10 cells that penetrated the mouse bladder membrane.[3, 4)] Although both are highly metastatic sublines, B16-F10 cells preferentially metastasize to the lung following intravenous injection, while B16-BL6 cells metastasize to the lung after subcutaneous injection. Regarding hematogenous metastasis, B16-F10 cells are much more metastatic than B16-BL6 cells.[3)] TI-241 was one of the four isolated genes that were highly expressed in B16-F10 cells but less so in B16-BL6 cells and even less in low-metastatic B16-F1 cells.[5)] TI-241 is a mouse homologue of a transcription factor, human ATF3 and rat LFR-1, which belongs to the Jun-Fos family. Since transfection of TI-241 into B16-F1 cells confers high-metastatic potential after intravenous inoculation, it is possible that TI-241 regulates the expression of various genes involved in the complex process of metastasis, especially after vessel invasion, namely cell attachment to and invasion/growth at the target organs.[5)] We looked next at the application of this gene in cancer therapy, particularly antisense strategies involving oligonucleotides. Since phosphorothioate oligonucleotides were reported to have a nonspecific growth-inhibitory effect on melanoma cells, we investigated the effect of an antisense oligonucleotide of ATF3 on human colon cancer cell line HT29.[6)]

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MATERIALS AND METHODS

Cell lines and oligonucleotides HT29 cells and human fibroblast cells were cultured in RPMI supplemented with 5% heat-inactivated calf serum. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The sense (AAAACTAGGCAATGTACTCTTCC) and antisense (GGAAGAGTACATTGCCTAGTTTT) phosphorothioate oligonucleotides were purchased from Toagosei, Japan. Blast nucleotide sequence analysis revealed no significant homology with known sequences. The oligonucleotides were dissolved in sterile, double-distilled water and added to the medium. For growth assay, the cell suspension (1×10^4/250 μl) was added to each well (24-well plates).

Western blot analysis To prepare whole-cell extracts of HT29 cells, 5×10^5 cells were washed twice with ice-cold

phosphate-buffered saline (PBS), trypsinized and pelleted by centrifugation at 4000 rpm for 2 min at 4°C. Pellets were resuspended and lysed in 50 μl of RIPA-2 buffer (10 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.1% NP40, 0.1% sodium deoxycholate (DOC), supplemented with protease inhibitors), after which the lysates were cleared by sonication. Proteins (1×10^5 cells) resolved by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to a polyvinylydene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA), followed by incubation of the membrane with a rabbit polyclonal IgG directed against the 19 C-terminal amino acids of human ATF3 (Santa-Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature. Specifically bound antibodies were detected by incubation with horseradish peroxidase-conjugated anti-rabbit serum for 1 h at room temperature and visualized by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions.

Adhesion assay To each well of collagen-coated 24-well plates was added 1 ml of the cell suspension (1×10^5/ml) pretreated without or with oligonucleotides (20 μM) for 60 h. The cells were allowed to adhere at 37°C in a humidified atmosphere of 5% CO_2. After 60 min, unbound cells were removed by washing the wells with 1 ml of PBS. Adherent cells were detached with 100 μl of trypsin-EDTA and were counted under a microscope. The results are representative of three independent experiments in triplicate.

Invasion assay Biocoat Matrigel invasion chambers (8-μm pore size) were purchased from Becton Dickinson Labware (Mountain View, CA). To each well of 24-well plates was added 750 μl of the human fibroblast suspension (5×10^4/750 μl). After 60 h, 500 μl of HT29 cells (5×10^4/500 μl of RPMI medium supplemented with 1% heat-inactivated calf serum and 0.1% bovine serum albumin (BSA)) pretreated with oligonucleotide (20 μM) or without oligonucleotide were added to each chamber. Chambers were incubated at 37°C for 24 h. Noninvasive cells were removed from the upper surface of the membrane with a cotton swab. Invasive cells on the underside of the membrane were counted under a microscope (250× magnification) in three different fields, after 70% ethanol fixation and Wright-Giemsa staining. The results are representative of three independent experiments in triplicate.

***In vivo* assay** HT29 cells cultured with and without oligonucleotide (20 μM) were harvested with trypsin/EDTA (0.25%/0.02%) and washed twice with ice-cold PBS. The cells (5×10^5) were suspended in 50 μl of ice-cold PBS with and without 10 nmol oligonucleotide, then inoculated subcutaneously into the Balb/nude mice (6-week-old females, Charles River Japan, Tokyo). Six mice were used for one group. Ten, 20 and 30 days after the first inoculation, each mouse was administered 10 nmol oligonucleotide or saline to the tumors. The tumor volumes were measured using the formula length (mm) × width (mm) × depth (mm).

RESULTS

To examine the effect of the antisense oligonucleotide on the 3′ region of *ATF3* gene, we cultured HT29 cells (20 μM) for 60 h. The antisense oligonucleotide showed a clear inhibitory effect on the expression of ATF3 compared with the sense oligonucleotide group and untreated control (Fig. 1). Two bands of about 26 kDa were detected, and both were thought to show ATF3 activity.[7] The time-course study revealed the inhibitory effect of the antisense oligonucleotide to be maximal at 60 h. The same inhibitory effect of the antisense oligonucleotide was observed when compared with the random oligonucleotide (data not shown). To determine the oligonucleotide effects on growth of HT29 cells *in vitro*, we cultured the cells with sense and antisense oligonucleotides (Table Ia). There was no significant growth difference at 60 h, and the calculated doubling time was about 20 h for all. The effects of antisense oligonucleotide on the two most important factors for ectopic growth, cell adhesion and invasion, were then studied. The antisense oligonucleotide inhibited cell adhesion to the collagen-coated floors of the plates, compared with untreated cells and cells treated with the sense oligonucleotide (Table Ib). With the antisense oligonucleotide, the number of attached cells was about half that of the other two control groups. Also the antisense oligonucleotide inhibited cell invasion to the Matrigel of the chamber floor, compared with untreated cells and cells treated with the sense oligonucleotide (Table Ic). There was a significant inhibitory effect with the antisense oligonucleotide and the number of invasive cells treated with antisense oligonucleotide was less than one-third that of the other two control groups. Human colon cancer cells, including HT-29 cells, were reported to metastasize to the skin by intravenous inoculation, and subcutaneous growth was necessary in the process of tumor metastasis.[8] Since

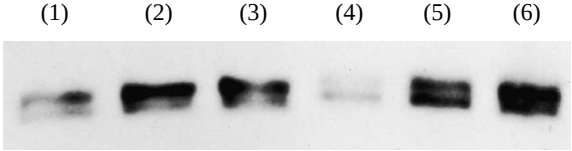

Fig. 1. Western blot analysis of ATF3 expressed in HT29 cells cultured with 20 μM antisense oligonucleotide (1, 4), sense oligonucleotide (2, 5), and without oligonucleotides (3, 6). Cells were cultured for 30 h (1, 2, 3) and 60 h (4, 5, 6). Whole-cell extracts (1×10^5 cells/lane) were resolved by SDS-PAGE and subjected to western blot analysis with ATF3 antiserum.

Table I. The Effects of Oligonucleotides on the HT29 Cells

a) The effect of oligonucleotides on the proliferation of HT29 cells *in vitro*

Oligonucleotides cultured with HT29 cells	Cell number ($\times 10^4$)		
	20 h	40 h	60 h
none	2.26±0.61	6.30±0.85	16.0±2.27
sense	2.48±0.56	6.63±1.72	17.2±2.27
antisense	1.33±0.20	6.70±0.82	15.8±1.51

To each well of 24-well plates was added cell suspension (1×10^4 cells/250 μl medium) and 20 μM oligonucleotide. The cells were detached and counted after 20, 40 and 60 h.

b) Cell adhesion assay

Oligonucleotides cultured with HT29 cells	Attached cell number ($\times 10^4$)
none	4.1±0.1
sense	3.7±0.5
antisense	2.1±0.2 ($P<0.05$)

To each well of collagen-coated 24-well plates was added cell suspension (1×10^5/1000 μl medium) pretreated with 20 μM oligonucleotide or without oligonucleotide for 60 h. After 60 min, nonadherent cells were removed, then adherent cells were detached with 100 μl of trypsin-EDTA and counted under a microscope.

c) Cell invasion assay

Oligonucleotides cultured with HT29 cells	Invasion cell number (cell count/field)
none	15.0±3.0
sense	9.0±1.8
antisense	3.2±0.4 ($P<0.05$)

To each well of 24-well plates was added human fibroblasts (5×10^4/750 μl of medium). After 60 h, 5×10^4 HT29 cells in 500 μl of RPMI medium supplemented with 1% heat-inactivated calf serum and 0.1% BSA were added to each chamber. After 24 h, the invasive cells on the underside of the membrane were counted under a microscope (250× magnification) in three different fields.

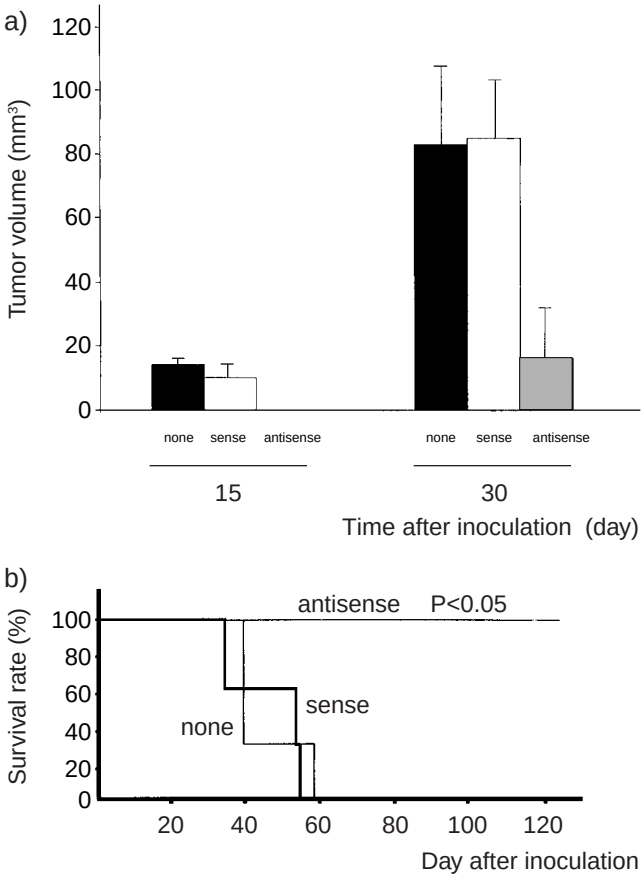

Fig. 2. a) The tumor volume of mice inoculated subcutaneously with HT29 cells (5×10^5). HT29 cells cultured with and without oligonucleotides (20 μM) for 60 h were harvested with trypsin/EDTA (0.25%/0.02%) and washed twice with ice-cold PBS. After the tumor formation, 10 nmol of oligonucleotide was administered three times to the tumors (10, 20 and 30 d after the first inoculation). Tumor volume was measured 15 and 30 d after the first inoculation. b) The survival curves of the above mice.

HT29 is not suitable for *in vivo* experimental metastasis studies because of its low metastatic potential, we adopted a subcutaneous inoculation model and studied the effects of ATF3 antisense oligonucleotide on HT29 cells in Balb/nude mice inoculated subcutaneously. In the process of subcutaneous growth, the interaction with the matrix and invasion are thought to be important factors. Adhesion and invasion experiments are thought to reflect these factors. As shown in Fig. 2a, the antisense oligonucleotides inhibited tumor growth of HT29 cells inoculated subcutaneously. The mice inoculated with HT29 cells and treated with the sense oligonucleotides or saline all died within 60 days. The main cause of death was peritoneal dissemination. But the mice inoculated with HT29 cells and treated with antisense oligonucleotide survived over 120 days (Fig. 2b).

DISCUSSION

ATF3 is a 21-kDa leucine zipper-containing protein that belongs to the Jun-Fos family. ATF3 functions as the homodimer or heterodimeric complexes with c-Jun and Jun B, by binding to CRE, AP-1, and ATF sites.[9, 10] Recently, ATF3 was reported to be negatively regulated by the endogenous growth arrest gene gadd153/Chop10, but most of its functions are still unknown.[11] Since the relationship between the expression level of Jun-Fos family proteins and their metastatic potential had been reported, we postulated that ATF3 may regulate the expression of sev-

eral metastasis-associated genes.[12, 13] Therefore, we assumed that the treatment of the cells with antisense oligonucleotide of ATF3 might control some factors that influence ectopic growth potential. The present report shows that the antisense oligonucleotide designed against a region near the 3′ end inhibited cell adhesion and invasion of HT29 cells. The relationship between expression of ATF3 and cell adhesion/invasion has not been reported, except for its activation of the E-selectin promoter.[14] As mentioned above, cell attachment and invasion to the target organs are thought to be the most important factors for ectopic growth in the process of metastasis. These two factors might explain why the antisense oligonucleotide inhibited the growth of HT29 cells *in vivo*. Our results showed that ATF3 plays an important role in the ectopic growth/metastasis of HT29 colon cancer cells and antisense oligonucleotide could inhibit its expression.

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