Antisense Oligodeoxynucleotide Targeted to Midkine, a Heparin-binding Growth Factor, Suppresses Tumorigenicity of Mouse Rectal Carcinoma Cells 1

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ABSTRACT

Midkine (MK), a heparin-binding growth factor, is overexpressed in a wide range of human carcinomas and is believed to contribute to tumorigenesis and tumor progression. To develop an antimurine reagent, we designed a phosphorothioate antisense oligodeoxynucleotide molecule based on the secondary structure of MK mRNA. The antisense MK at the dosage of 5 μM suppressed MK production by CMT-93 mouse rectal carcinoma cells after cationic liposome-mediated transfection, to 13% of that in control cultures. The growth of CMT-93 cells and their colony formation in soft agar were inhibited by the addition of the antisense MK, whereas the control reagent, the sense MK, showed no effects. On s.c. injection into nude mice, CMT-93 cells transfected with the antisense MK formed tumors much smaller than those by control cells. Finally, untreated CMT-93 cells were inoculated to nude mice, and 7 days later the antisense MK (50 μM) with atelocollagen was directly injected into the preformed tumor region to evaluate the curative effect; the injection was repeated at the interval of 2 weeks. During the period of 10–41 days after initiation of therapy, the rate of increase of tumor volume treated with the antisense MK was found to be about 4.2-fold lower than that seen after treatment with the sense MK. On this occasion, proliferation of tumor cells as estimated by 5-bromodeoxyuridine incorporation was strongly inhibited, whereas angiogenesis was less affected. These findings strongly suggested the usefulness of MK antisense oligodeoxynucleotide as a new reagent for cancer therapy.

INTRODUCTION

Overexpression of growth factors and components of their signal transduction systems is observed frequently in carcinomas and serves as a principal cause of carcinogenesis in many cases (1). Thus, growth factors and their receptors have become one of the major targets to develop new modes of therapy for carcinoma. Antisense ODNs,1 antisense RNA, or ribozymes targeting these molecules have yielded beneficial results to suppress tumor growth both in vitro and in vivo. Typical examples are vascular endothelial growth factor, basic fibroblast growth factor and its receptor in melanoma (2, 3), and insulin-like growth factor-I receptor, hepatocyte growth factor, and tumor necrosis factor in glioblastoma (4–6). However, growth factor-targeted therapy of malignancy is still at the initial stage. The range of malignancies that can be treated is restricted, and injection of reagents after tumor formation has been effective in only a few cases (3, 7).

MK, a heparin-binding growth factor (8, 9), appears to be an excellent target of cancer therapy. MK is a Mr 13,000 protein rich in basic amino acids and cysteine (8, 9), and shares ~50% sequence identity with another heparin-binding growth factor, PTN (10), also called heparin-binding growth associated molecule (11). MK is over-expressed in a variety of tumors such as esophageal, gastric, colon, pancreatic, hepatocellular, lung, breast, and urinary bladder carcinomas (12–17), neuroblastoma (15) and Wilms’ tumor (12), whereas in normal adult tissue, its expression is usually low or undetectable (10, 12). On transfection of the cDNA, MK transforms NIH3T3 cells (18). MK promotes survival (19, 20), growth (21, 22), and migration (23–25) of many cells. These activities in combination are believed to contribute to oncogenesis and tumor progression. Indeed, the affinity-purified antibody to MK partly suppressed growth of Wilms’ tumor cells in culture (22). The present study was performed to develop a more potent antitumor reagent. For this purpose, we designed MK antisense ODNs and examined their antitumor activity against a rectal carcinoma cell line with particular attention to treatment of established tumors by intratumoral injection. For in vivo treatment, we adopted a new gene transfer method using a biomaterial, A. collagen, prepared from bovine dermal collagen (26), because antisense effects with the aid of cationic lipids in culture are not always reproducible in vivo (27).

MATERIALS AND METHODS

ODNs. Phosphorothioate-modified ODNs were synthesized with an automated solid-phase nucleotide synthesizer (Expdite8900 Nucleic Acid Synthesis System; Applied Biosystems) and subsequently purified using a Wako Handyl ODS column (Waters). MK antisense ODNs corresponding to different regions of mouse MK coding sequence were synthesized. The sequence and location of each ODN in MK cDNA (Ref. 2; Fig. 1) were as follows: antisense-1, 5’-AAGAACCTGCTCCGCTGAT-3’ (bases 1 to 20); AS, 5’-AGGGCGAAAGAAGGAAAG-3’ (bases 15 to 32); antisense-3, 5’-CTCCCAATATCCTTCTTCC-3’ (bases 218 to 235); and antisense-4, 5’-GGGCTTATGCTACCGGAT-3’ (bases 349 to 366). Sense and reverse ODNs for AS were also designed as controls: SEN, 5’-CTTCCTCCTTCTCCTGCCCT-3’; and REV, 5’-GAAAGAACGGACAGCGGA-3’.

Cell Culture Conditions and Transfection of ODNs. CMT-93 cells (American Type Culture Collection, Rockville, MD) derived from mouse rectal carcinoma were maintained in DMEM with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO2. Cells were inoculated at a density of 3 × 105 cells/ml in RPMI medium in a 5% CO2 atmosphere at 37°C. After incubation for 12 h, the medium was changed to DMEM with 10% FBS. For transfection, the cells were washed and incubated in fresh medium for 1 h. ODNs were then added to the medium, and the mixture was left to incubate for 4 h. After incubation, the cells were washed with PBS, and fresh DMEM with 10% FBS was added. After incubation for 4 h, the transfection medium was discarded, and the cells were washed with PBS again. The medium was renewed with DMEM with 10% FBS, and the cells were incubated for 24 h.

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3 The abbreviations used are: ODN, oligodeoxynucleotide; MK, midkine; PTN, pleiotrophin; FBS, fetal bovine serum; BrdUrd, 5-bromodeoxyuridine; A. collagen, atelocollagen; SEN, sense control for AS; REV, reverse control for AS; AS, Antisense-2.
trypsinization and were used. For Western blotting analysis and ELISA, the medium was then replaced with fresh DMEM containing insulin (10 μg/ml), transferrin (5.5 μg/ml), sodium selenite (6.7 ng/ml), and heparin (20 μg/ml). After 16 h of incubation, conditioned medium was collected for analysis.

**Proliferation Analysis.** AS- and SEN-transfected CMT-93 cells were plated in 24-well plates in DMEM supplemented with 10% FBS at a density of 3 × 10^4 cells/well. Later (3 h), the medium was changed to serum-free medium, and the cells were cultured for ±5 days. Cell proliferation was monitored using a cell counting kit (Dojink; Kumamoto, Japan). Chemically synthesized human MK (5 ng/ml; Peptide Institute, Osaka, Japan) was added to some AS-transfected cultures.

**Colony Formation in Soft Agar.** AS-, SEN-, or REV-transfected CMT-93 cells were suspended in 0.33% Noble agar (Sigma Chemical Co.) dissolved in 1 ml of serum-free medium and plated on top of a 1.5-ml base layer of 0.5% agar in the same medium in 35-mm dishes. After 9–11 days of incubation at 37°C in an incubator under a humidified 5% CO2 atmosphere, the numbers of colonies formed in the whole dish were counted. Chemically synthesized MK (10 ng/ml or 50 ng/ml) was exogenously added as a rescue molecule to both base and top layers of some AS-transfected cultures.

**In Vivo Tumorigenicity Study.** A total of 9 × 10^4 AS- or SEN- (5 μM) pretransfected CMT-93 cells were s.c. inoculated in 0.3 ml of serum-free medium through a 24-gauge needle into both lower flanks of 8-week-old athymic nude mice obtained from SLC (Tokyo, Japan). One week after inoculation, AS or SEN plus A. collagen (Koken Co. Ltd., Tokyo, Japan) complex (total volume 50 μl/mouse) kept at 4°C were directly injected into the tumor region. The final concentration of A. collagen was 1.75% and that of anti-AS were transferred with 5 μg/ml. To determine intratumor microvessel density, the five vascular areas within a section were selected and images were obtained with 200-fold magnification (microscope field: 666 μm × 666 μm, 0.444 mm^2) under a confocal microscope and computer system. Microvessels in each image of five areas were independently counted on a computer display, and the arithmetical mean in each area was used to calculate the mean intratumor microvessel density for each tumor section.

**Statistical Analysis.** The data were analyzed using the Mann-Whitney U test, and probability values <0.05 were considered to indicate significant differences.

**RESULTS**

**Effects of MK Antisense ODNs on CMT-93 Carcinoma Cells in Culture.** We predicted the secondary structure of mouse MK mRNA by computer analysis (Genetyx-Mac. Version 10); the algorithm for the calculation was that of Zuker and Stiegler (34). We designed MK antisense ODNs to target loop regions of the mRNA (Fig. 1). Of the four antisense ODNs synthesized, only AS suppressed synthesis and secretion of MK, as determined by the Western blotting analysis and ELISA.

**In Vitro Tumorigenicity Study.** A total of 9 × 10^4 AS- or SEN- (5 μM) pretransfected CMT-93 cells were s.c. inoculated in 0.3 ml of serum-free medium through a 24-gauge needle into both lower flanks of 8-week-old athymic nude mice obtained from SLC (Tokyo, Japan). One week after inoculation, AS or SEN plus A. collagen (Koken Co. Ltd., Tokyo, Japan) complex (total volume 50 μl/mouse) kept at 4°C were directly injected into the tumor region. The final concentration of A. collagen was 1.75% and that of each ODN was 50 μg/ml. To determine intratumor microvessel density, the five vascular areas within a section were selected and images were obtained with 200-fold magnification (microscope field: 666 μm × 666 μm, 0.444 mm^2) under a confocal microscope and computer system. Microvessels in each image of five areas were independently counted on a computer display, and the arithmetical mean in each area was used to calculate the mean intratumor microvessel density for each tumor section.

**Statistical Analysis.** The data were analyzed using the Mann-Whitney U test, and probability values <0.05 were considered to indicate significant differences.
examined the effects of AS on tumor formation by these cells. CMT-93 cells were treated with AS or SEN with the aid of LipofectAMINE-plus and were s.c. inoculated into male nude mice. After inoculation (7 days), tumor formation was clearly observed by SEN-pretreated cells, whereas barely visible tumor was formed by AS-pretreated cells (Fig. 4A). At that time, additional AS or SEN was injected with A. collagen but without LipofectAMINE-plus. In animals injected with SEN-pretreated cells, massive and angiogenic solid tumors were observed, whereas the AS-pretreated cells yielded much smaller tumors (Fig. 4A). This reduced tumor growth was dependent on the dose of AS injected (Fig. 4B), and 40-μM AS inhibited tumor growth by 80% as compared with SEN-treated cells (Fig. 4B).

Treatment of Preformed Tumors by MK Antisense ODN. We then injected preformed tumors with AS. Untreated CMT-93 cells were inoculated into nude mice. After inoculation (7 days) when a palpable tumor was formed, SEN or AS with or without A. collagen was injected into the tumor, and the injection was repeated every 14 days. LipofectAMINE-plus was not used in this series of experiments. AS with A. collagen markedly suppressed tumor growth as compared with SEN with A. collagen (P < 0.001; Fig. 5A). The rate of increase of tumor volume treated with AS with A. collagen was ~4.2-fold lower than that seen after treatment with SEN with A. collagen 10–41 days after initiation of tumor therapy (Fig. 5A). AS injection without A. collagen was less effective, although AS alone also delayed tumor growth as compared with SEN alone (P < 0.01; Fig. 5A). SEN with A. collagen scarcely inhibited tumor growth as compared with SEN alone or PBS injection (Fig. 5A), indicating that A. collagen alone does not have antitumorigenic activity to CMT-93 cells. This point was directly examined by injecting A. collagen to CMT-93 tumor (Fig. 5B). Indeed, the A. collagen-injected tumors exhibit growth properties indistinguishable from untreated tumors (Fig. 5B).

In the above experiment, tumor growth was monitored by determining tumor volume estimated with calipers. At the end of the study (41 days after initiation of tumor therapy), all of the animals were sacrificed, and tumor weight was determined. AS with A. collagen significantly suppressed the increase of tumor weight as compared with controls (Fig. 5C). Furthermore, we found that intratumor injection of AS with A. collagen resulted in significant reduction of tumoral MK content as determined by ELISA in accordance with their therapeutic effects (Fig. 5D).
We evaluated whether AS exhibited antiangiogenic effects to suppress tumor growth in vivo. Ten and 17 days after initiation of therapy, intratumoral microvessel density was slightly but significantly suppressed by AS with A. collagen as compared with SEN with A. collagen (Fig. 6). On the other hand, cell division monitored immunohistochemically by incorporation of BrdUrd was markedly suppressed by treatment with AS with A. collagen as compared with treatment with SEN with A. collagen (Fig. 7); the number of dividing cells in the former group was ~20% of that in the latter at both 10 and 17 days after initiation of therapy. The degree of suppression of cell division in vivo correlated well with the degree of suppression of tumor growth rate. However, no significant differences were observed in the degree of apoptosis determined by the terminal deoxynucleotidyl transferase-mediated nick end labeling method (32) between tumors treated with AS with A. collagen and those treated with SEN with A. collagen (data not shown).

Fig. 6. Decreased vessel density in tumors injected with AS with A. collagen. Histological sections from tumors injected with AS or SEN mixed with A. collagen were immunostained for endothelial cells with anti-CD31 antibodies. We examined four excised tumors on both day 10 and 17 after therapy and intratumor microvessel density (vessels/mm²) was determined. Results represent the means (n = 4 tumors); bars, ± SD. #, P < 0.05 versus SEN + A. collagen.
expression of both factors may be required when they are coexpressed in the tumor. Because CMT-93 cells used here did not express PTN, these cells are a suitable system in which to evaluate the effects of MK-directed therapy without consideration of PTN.

Successful treatment of tumors with MK antisense ODN was achieved not only by selecting a suitable molecule, MK, but also by choosing suitable delivery conditions using A. collagen. A. collagen achieved not only by selecting a suitable molecule, MK, but also by ornithine decarboxylase-directed therapy without consideration of PTN.

In conclusion, we demonstrated potent growth inhibitory effects of MK antisense ODN on rectal carcinoma growth in vivo. Because strong expression of MK is not observed frequently in adult tissue (8, 12–17), MK-directed therapy is expected to show stringent tumor specificity with low side effects. Much work remains to be performed before MK antisense ODN can be applied to treatment of human cancer. Specifically, tumor suppressive effects of the reagent should be examined in other types of tumors to define the scope of MK-directed therapy.

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REFERENCES


Y. Takei, unpublished observations.


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Fig. 7. BrdUrd immunostaining. Tumor-bearing mice were pulsed with BrdUrd 16 h before sacrifice to allow the uptake of BrdUrd by proliferating cells. Multiple tumor sections sampled at various time points were stained and analyzed by confocal microscopy. Tumor samples were from groups of mice injected with SEN (A and C) with A. collagen or AS (B and D) with A. collagen both 10 (A and B) and 17 (C and D) days after initiation of therapy. Bar, 50 μm. E, decrease in number of BrdUrd-positive cells in tumors injected with AS with A. collagen. Results (BrdUrd-positive cells/5 field at ×200) represent the means (n = 4 tumors); bars, ± SD. *P < 0.001 versus SEN + A. collagen.

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