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

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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 antitumor 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 signaling systems is observed frequently in carcinomas and serves as the 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,3 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 M₄₃,000 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 overexpressed 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 (Expedite8900 Nucleic Acid Synthesis System; Applied Biosystems) and subsequently purified using a Wako V Bond 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. 28; Fig. 1) were as follows: antisense-1, 5’-AAGAAGGCGCCTCTGCTGCAT-3’ (bases 1 to 20); AS, 5’-GGGCGGAGAAGAAGAAG-3’ (bases 15 to 32); antisense-2, 5’-CTCCTATCTTCTCTTCC-3’ (bases 218 to 235); and antisense-3, 5’-GGCTTGTGATCCACCCG-3’ (bases 349 to 366). Sense and reverse ODNs for AS were also designed as controls: SEN, 5’-CTCCTATCTTCTTCCCTC-3’; and REV, 5’-GAAGAGGAGAAGGCGGGA-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/35-mm tissue culture dish and cultured under the following conditions: 5% of each ODN stock solution (1 mm) and the plus reagent (10 μl) were mixed in DMEM (85 μl) in a small, sterile tube. After immediate mixing with a Vortex mixer and standing at room temperature for 15 min, the LipofectAMINE reagent (4 μl) in DMEM (100 μl) was added, and the mixture was left at room temperature for 15 min. Then, 0.8 ml of DMEM was added to generate ODN-transfection complex. Medium was removed from the culture of CMT-93 cells, and the ODN-transfection complex, the total volume of which was 1 ml, was added. After incubation for 3 h at 37°C, 1 ml of DMEM with 10% FBS was added, and incubation was continued for 4 h. For proliferation analysis, colony formation in soft agar, and in vivo tumorigenicity study, the transected cells were recovered by

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5 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.
A. collagen was 1.75% and that of each ODN was 50

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 (Dojin; Kumamoto, Japan). Chemically synthesized MK (5 ng/ml; Peptide Institute, Osaka, Japan) was added to several 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% CO_2 atmosphere, the numbers of colonies formed in the whole dish 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.

In Vivo Tumorigenicity Study. A total of 9 × 10^6 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 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. Tumor diameters were measured at regular intervals by calipers, and tumor volume in mm^3 was calculated by the formula: volume = (length)^2 × length/2 (29). Data are presented as means ± SE.

Tumor Therapy. A total of 1.5 × 10^6 untransfected CMT-93 cells were s.c. inoculated as described above. After 7 days when tumors reached an average volume of ~50 mm^3, the tumor-bearing nude mice were randomly divided into five different treatment groups (AS alone, SEN alone, AS plus A. collagen, SEN plus A. collagen, and PBS). AS or SEN with or without A. collagen was directly injected into the tumor region. The final concentration of A. collagen was 1.75% and that of each ODN was 50 μg/ml. In positive controls, PBS alone was injected. Each therapeutic reagent was injected into the tumors every 2 weeks after the first injection. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine.

Western Blotting Analysis and ELISA. Proteins in conditioned medium were separated by electrophoresis on 15% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes (18). Blots were blocked with 5% nonfat dried milk and incubated with rabbit anti-MK antibody (18) and horseradish peroxidase-conjugated goat anti-rabbit IgG. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia). Quantitative analysis of the blots was performed with an imaging densitometer. The amount of MK was determined by ELISA as described previously (30).

BrdUrd Treatment. For BrdUrd labeling, mice were injected i. p. with 200 μg of BrdUrd 16 h before sacrifice as described by Marzo et al. (7).

Immunohistochemistry. Parts of the tumor tissues were snap-frozen in liquid nitrogen and kept frozen at −80°C until use. Sections, 2-μm thick, were cut with a cryostat and fixed in acetone. Sections were then stained with rat antihuman CD31 antibody (Pharmingen).

For BrdUrd immunostaining, the DNA of the tissue sections was denatured by incubation with 2 M hydrochloric acid for 10 min. To neutralize the pH, sections were rinsed in 0.1 M sodium tetraborate for 10 min followed by rinsing in PBS (31). The sections were then incubated with anti-BrdUrd rat monoclonal antibody (Oxford Biotechnologies Ltd.).

Specific antibody staining was visualized using FITC-labeled goat antirat IgG (Cappel, Durham, NC). The diluent of FITC-labeled secondary antibody was filtered (0.22 μm) before use to prevent nonspecific staining by contamination with FITC debris. After washing with PBS, all of the sections were mounted with a ProLong Antifade kit (Molecular Probes) and examined by confocal microscopy (MRC 1024; Bio-Rad).

Apoptotic cells were estimated using Apoptosis Detection kit (Takara Shuzo Co., Ltd., Shiga, Japan) based on the terminal deoxynucleotidyl transferase-mediated nick end-labeling method (32).

Intratumor Microvessel Density Assessment. Microvessel counting of CD31 (PECAM-1)-positive vessels was performed as described by Weidner et al. (33). To determine intratumor microvessel density, the five vascular areas within a section were selected and images were obtained with 200-fold magnification (microscopy 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 in CMT-93 rectal carcinoma cells after transfection with the aid of LipofectAMINE-plus (Fig. 2A). SEN and REV ODNs for AS showed no effects (Fig. 2B). Densitometric analysis of the blots revealed that 5 μM of AS suppressed MK production to 13% of

![Graph](https://example.com/graph.png)
that in control cultures. ELISA estimation of MK in culture medium confirmed these results; the values were 218, 1439, and 1647 pg/ml for AS, REV, and SEN, respectively.

When AS was added to cultures of CMT-93 cells, it inhibited their growth especially 4–5 days after addition. However, SEN showed no such effect (Fig. 3A). Addition of 5 ng/ml of MK abolished the effect of AS, confirming that the effect of AS was specific. Because the level of MK in the medium of untreated cells was about 1.5–1.8 ng/ml, the amount of added MK was not much excessive.

Colonies formation in soft agar, a property closely associated with malignancy (35), was also suppressed by AS but not by SEN or REV (Fig. 3B). Exogenously added MK also counteracted AS on colony formation (Fig. 3B). That a higher concentration of MK (50 ng/ml) was required for the attenuation in this system may be explained by the interaction of MK with agar.

Effects of MK Antisense ODN on Tumor Growth in Nude Mice. Because AS specifically inhibited production of MK in CMT-93 cells and inhibited their growth and colony formation in soft agar, we 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).
SEN/H11001

MK in each supernatant was measured by ELISA specific for MK. Therapy) were homogenized in ice-cold Tris-buffered saline containing 1% Triton X-100. 50 mm3. Tumor diameters were measured at a regular interval for up to 41 days with experiments were performed as in A. collagen alone (50 μl) was injected to the tumors (E); the controls were untreated tumors (F). Day 0 corresponds to 7 days after inoculation of cells, when tumor volume was ~50 mm3. Tumor diameters were measured at a regular interval for up to 41 days with calipers and tumor volume was calculated. Results represent the means (n = 6); bars, ± SE. * P < 0.001 versus SEN + A. collagen; # P < 0.01 versus SEN alone. B, effects of A. collagen on tumor growth. On days 0 and 14, A. collagen alone (50 μl) was injected to the tumors (E); the controls were untreated tumors (F). Day 0 corresponds to 7 days after inoculation of CMT-93 cells. Tumor diameter was determined, and the result was expressed by ratio of tumor volume to that in day 0. Results represent the means (n = 4); bars, ± SE. C, tumor weights. Experiments were performed as in A. The mice were sacrificed 41 days after injection, all tumors were excised, and the tumor weights were determined. Results represent the means (n = 6); bars, ± SE. * P < 0.001 versus SEN + A. collagen; # P < 0.01 versus SEN alone. D, MK contents in tumors. Experiments were performed as in A. The excised tumors (on days 10, 17, and 24 after therapy) were homogenized in ice-cold Tris-buffered saline containing 1% Triton X-100 and protease inhibitor mixture (Sigma Chemical Co.) and then centrifuged. The amount of MK in each supernatant was measured by ELISA specific for MK. * P < 0.001 versus SEN + A. collagen. Results represent the means (n = 4); bars, ± SD.

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 deoxynucleotidyld 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).

**DISCUSSION**

An antisense ODN to MK successfully suppressed synthesis of MK in CMT-93 rectal carcinoma cells leading to inhibition of their growth in culture and colony formation in soft agar. Furthermore, tumor formation by the treated cells in nude mice was markedly inhibited. Even injection of the antisense ODN into preformed tumor tissue suppressed tumor growth significantly. These results clearly established that MK participates in growth of tumor cells both in vitro and in vivo. Although MK is known to be an angiogenic factor (36), angiogenesis in tumors treated with AS with A. collagen was only slightly inhibited in this occasion. In contrast, AS with A. collagen strongly inhibited cell division of the tumor cells in vivo. Thus, we considered that MK antisense ODN inhibited growth of the tumor principally by its anticlell proliferation activity with some contribution of the antiangiogenic activity.

Treatment of pregrown tumors by injection with antisense or ribozyme reagents to growth factors or receptors has been successful in only a few cases (3, 7). Targeting of basic fibroblast growth factor and its receptor markedly suppressed melanoma growth in nude mice through prevention of angiogenesis (3). Growth of mesothelioma in nude mice was suppressed by transforming growth factor β-2 antisense ODN (7). The results of the present investigation strongly suggested that MK-directed therapy is a promising method for treatment of malignancy. The most attractive point of MK-targeted therapy is preferential expression of MK in tumors (12–17). The tumor-associated expression of MK also enabled the usage of the MK-promoter to mediate tumor-specific expression of suicide genes (37, 38). Serum MK level has recently been introduced as a tumor marker elevated in a wide variety of malignancies (39). Therefore, MK is a molecule with multiple implications in clinical oncology.

PTN, which has ~50% sequence identity with MK, has potent tumorigenic potential in NIH3T3 cells (40). Transfection with a ribozyme-cleaving PTN mRNA has been shown to suppress growth of pancreatic carcinoma, chloiocarcinoma, and melanoma (41–43), whereas treatment of preformed tumors with the ribozyme has not been reported. Recent studies have revealed components of signaling receptors for MK and PTN (24, 44–50), and the downstream signaling systems (19, 50–52). Much of the identified signaling molecules are shared between MK and PTN; they are receptor-like protein tyrosine phosphatase ε (24, 46, 50), syndecans (44, 45), phosphatidylinositol 3’-kinase (17, 50, 51), and extracellular signal-regulated kinase (17, 50, 51). Because of the similar biological activities of MK and PTN (53) and their shared signaling molecules, simultaneous inhibition of

![Fig. 5. Antitumor effect of AS in CMT-93 tumor-bearing nude mice. A, tumor growth curves. On days 0, 14, and 28, AS (△) or SEN (□) mixed with A. collagen, SEN alone (Y), SEN alone (□) or PBS alone (□) was injected into the tumor region, as indicated in the figure. Day 0 corresponds to 7 days after inoculation of cells, when tumor volume was ~50 mm3. Tumor diameters were measured at a regular interval for up to 41 days with calipers and tumor volume was calculated. Results represent the means (n = 6); bars, ± SE. * P < 0.001 versus SEN + A. collagen; # P < 0.01 versus SEN alone. B, effects of A. collagen on tumor growth. On days 0 and 14, A. collagen alone (50 μl) was injected to the tumors (E); the controls were untreated tumors (F). Day 0 corresponds to 7 days after inoculation of CMT-93 cells. Tumor diameter was determined, and the result was expressed by ratio of tumor volume to that in day 0. Results represent the means (n = 4); bars, ± SE. C, tumor weights. Experiments were performed as in A. The mice were sacrificed 41 days postinjection, all tumors were excised, and the tumor weights were determined. Results represent the means (n = 6); bars, ± SE. * P < 0.001 versus SEN + A. collagen; # P < 0.01 versus SEN alone. D, MK contents in tumors. Experiments were performed as in A. The excised tumors (on days 10, 17, and 24 after therapy) were homogenized in ice-cold Tris-buffered saline containing 1% Triton X-100 and protease inhibitor mixture (Sigma Chemical Co.) and then centrifuged. The amount of MK in each supernatant was measured by ELISA specific for MK. * P < 0.001 versus SEN + A. collagen. Results represent the means (n = 4); bars, ± SD.](cancerres.aacrjournals.org)
It has been reported that phosphorothioate ODNs interact and regulate the heparin-binding proteins including some growth factors and that the interaction is sometimes sequence specific (54). Contribution of such a mechanism to effects of antisense MK ODN is probably low, if any, because antisense MK ODN other than phosphorothioate ODN exhibited similar activities, and antisense MK ODN was considered to be slowly released and delivered to tumor cells, probably as a complex with A. collagen. However, occurrence of such action of phosphorothioate ODN (54) should be taken into account in future studies.

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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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); bar, ± SD. *P < 0.001 versus SEN + A. collagen.

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