Oligodeoxynucleotides modified at both 5′- and 3′-ends with inverted thymidine (5′-3′-inverted T) were introduced as new reagents for antisense strategies. These modifications were performed to make the oligodeoxynucleotides resistant to nucleases. The effectiveness of these oligodeoxynucleotides was evaluated in terms of inhibition of synthesis of midkine (MK), a heparin-binding growth factor, and consequent inhibition of growth of CMT-93 mouse rectal carcinoma cells. 5′-3′-Inverted T antisense MK suppressed synthesis of MK by CMT-93 cells and their growth in culture. Furthermore, 5′-3′-inverted T oligodeoxynucleotides exhibited less cytotoxicity and better stability than phosphorothioate oligodeoxynucleotides. When 5′-3′-inverted T antisense MK was mixed with atelocollagen, and injected into CMT-93 tumors pregrown in nude mice, tumor growth was markedly suppressed as compared with tumors injected with sense controls. The suppressive effect of 5′-3′-inverted T antisense MK on tumor growth was stronger than that of phosphorothioate antisense MK. These findings indicated the usefulness of inverted thymidine-modified antisense oligodeoxynucleotides as a new reagent instead of phosphorothioate-modified oligodeoxynucleotides.

Oligonucleotides have long been recognized to have huge potential as agents for turning off the expression of specific proteins, in most cases working by inducing degradation of the mRNA encoding the protein (1, 2). The possible therapeutic use of oligonucleotides as effective gene regulatory agents in antisense and antigenic approaches has kindled further interest in the development of oligonucleotide analogs (1, 3). Rapid degradation of the “natural” phosphodiester (PO) backbone oligonucleotides by nucleases (4, 5) necessitated chemical modification of the PO backbone. Chemical modifications such as methylyphosphonate (6, 7), phosphorothioate (PS) (8, 9), and phosphoramidate (10) oligonucleotides have been introduced to make the oligonucleotides stable to degradative enzymes in serum (11). Among these chemical modifications, PS-modified oligonucleotides are most frequently used because of their ease of manufacture, low cost, and resistance to nucleases. However, PS-modified oligonucleotides have been shown to have toxic side effects in both cells in culture and in animals (12–15).

Recently, several approaches have been employed to overcome this problem (16–20). The present study was performed to develop a new modification for production of antisense oligodeoxynucleotides (ODNs), which remain capable of inducing degradation of the target mRNA, are stable in serum, and exhibit less cytotoxicity. For this purpose, we adopted antisense ODNs modified with inverted thymidine at both 5′- and 3′-ends, designated as 5′-3′-inverted T antisense ODNs, according to recent reports (21, 22) on DNA enzymes modified with inverted thymidine at the 3′-end. Inverted thymidine modification at 5′- or 3′-ends is aimed to protect the ODNs against exonuclease attack. The utility of the new modification was evaluated by monitoring anti-cancer activity of midkine (MK) antisense ODN produced by the proposed method.

MK, a heparin-binding growth factor, is a 13-kDa protein rich in basic amino acids and cysteine (23, 24). MK is overexpressed in a variety of tumors, such as esophageal, gastric, colon, pancreatic, hepatocellular, lung, breast, and urinary bladder carcinomas (25–30), neuroblastoma (28) and Wilms’ tumor (25), and in normal adult tissue, its expression is usually low or undetectable (25, 31). These findings suggested that MK may be a suitable target for cancer therapy. Recently, we established PS-modified antisense ODN targeting MK, which blocked the growth of mouse rectal carcinoma cells (CMT-93) in vitro and in vivo, and indicated its possible usefulness for cancer therapy (32). Thus, the effects of 5′-3′-inverted T-modified ODNs and PS-modified ODNs were compared.

EXPERIMENTAL PROCEDURES
ODNs—ODNs modified with inverted thymidine at both 5′- and 3′-ends (5′-3′-inverted T) or at 3′-end (3′-inverted T) and PS-modified ODNs were synthesized with an automated solid-phase nucleotide synthesizer ( Expedite 8900 Nucleic Acid Synthesis System, Applied Biosystems) and subsequently purified using a Wakopak Handy ODS column ( Waters Associates). The ODNs showed single bands upon 20% polyacrylamide gel electrophoresis in 7 M urea (33). The target sequence for MK antisense ODN (core region, 18-mer) corresponded to the region of bases 15–32 of MK mRNA; the region was located shortly after the translation initiation site, and secondary structure prediction indicated that it was in the first loop region after ATG (32). The sequences of each antisense (AS) ODN used in this study were as follows (cf. Fig. 1): 5′-3′-inverted T AS, 5′-TGGGCGAGGAAGAAAGTG-3′; 3′-inverted
FIG. 1. Schematic representations of designed MK antisense oligonucleotides. A, chemical structures of PS-modified ODN and 5'-3'-inverted T-modified ODN. B, schematic representations of 5'-3'-inverted T AS, 3'-inverted T AS, PS AS, and 5'-3'-non-inverted T AS. Open boxes indicate the core sequence with the PO backbone, and the closed box indicates the core sequence with the PS backbone. The core sequence (18 bases) was as follows: AGGGCGAGAAGGAAG-GAAG.

The underlined Ts in the above sequences indicate inverted thymidine. Uppercase sequences are PO, and lowercase sequences are PS. We also used ODNs labeled with fluorescein isothiocyanate (FITC) at each 5'-end, which were synthesized and purified in a manner identical to the unlabelled one.

**Cell Culture Conditions and Transfection of ODNs**—CMT-93 cells (American Type Culture Collection, Manassas, VA) were derived from mouse rectal carcinoma were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2. Cells (3 x 105/35-mm tissue culture dish) were seeded and transfected for 3 h with ODNs in serum-free medium using LipofectAMINE PLUS (Inivitrogen) as described previously (32). The transfection complex contained 5 nmol of ODNs and 6.8 nmol of lipids. Then, 1 ml of DMEM with 10% FBS was added, and incubation was continued for 4 h. The medium was then replaced with fresh DMEM containing insulin (10 mg/ml), transferrin (5.5 mg/ml), sodium selenite (6.7 ng/ml), and heparin (20 μg/ml). After 16 h of incubation, conditioned medium was collected for analysis.

**Determination of the Size of Complex of ODNs and Liposomes**—The transfection complex (32) which contained ODNs, the plus reagent, and the LipofectAMINE reagent was used by FITC-labeled ODNs, and the complex was observed by confocal microscopy using an imaging densitometer (Bio-Rad 1024).

**Cytotoxicity Analysis**—Cells (2 x 105/well in a 24-well plate) were seeded in DMEM with 10% FBS and cultured overnight. Then, 5'-3'-inverted T SEN or PS SEN was added to cultures. The final concentration of ODNs was 0, 50, 100, and 200 μM as indicated in Fig. 3. Twenty-four and 48 h later, cell survival was monitored using a cell counting kit (Dojin, Kumamoto, Japan).

**Western Blotting Analysis**—Proteins in conditioned media were separated by electrophoresis on 15% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes (35). Blots were blocked with 5% nonfat dried milk and incubated with rabbit anti-MK antibody (35) and horseradish peroxidase-conjugated goat anti-rabbit IgG. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences). Quantitative analysis of the blots was performed with an imaging densitometer.

**Tumor Therapy**—A total of 1.5 x 106 untransfected CMT-93 cells were subcutaneously 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). After 7–9 days when tumors reached an average volume of ~60 mm3, the tumor-bearing nude mice were randomly divided into five different treatment groups (5'-3'-inverted T AS, 5'-3'-inverted T SEN, 3'-inverted T AS, PS AS, and PS SEN). All ODNs were dissolved in DMEM and mixed with an...
equal volume of atelocollagen (final concentration of atelocollagen, 1.75%) kept at 4 °C and injected directly into the tumor region as reported previously (32). The final concentration of PS SEN and AS was 50 μM and that of 5'-3'-inverted T AS and SEN and 3'-inverted T AS was 200 μM. Each therapeutic reagent (50 μl) was injected into the tumors every 2 weeks after the first injection. Animal experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine. Tumor diameters were measured at regular intervals with digital calipers, and tumor volume in mm^3 was calculated by the formula: volume = (length/2) × width/2 × height. All data are presented as means ± S.E.

**Animal Experiment with Cationic Liposome as a Delivery Reagent—**
Cationic liposome (LipofectAMINE PLUS) as a delivery reagent was examined in tumor therapy experiment. One hundred μl of 5'-3'-inverted T AS stock solution (1 μM) and the plus reagent (50 μl) were mixed in DMEM (100 μl). After immediate mixing with a Vortex mixer and standing at room temperature for 15 min, the LipofectAMINE reagent (25 μl) in DMEM (225 μl) was added, and the mixture was left at room temperature for 15 min. Fifty μl of the mixture was injected into the preformed tumor. On day 14, another injection was done, and tumor diameters were monitored as described above.

**Stability of 5'-3'-Inverted T-modified AS in FBS—** Each ODN was incubated in 5% FBS at 37 °C. Aliquots of the reaction were removed at different time intervals for electrophoretic analysis. Nuclease reactions were stopped by adding formamide gel loading buffer to each sample and heating at 95 °C for 10 min (33). All samples were then run on 20% polyacrylamide gels containing 7 M urea (Bio-Rad) and visualized by staining with a SYBR® Gold Nucleic Acid Gel Stain kit according to the manufacturer’s instructions (Molecular Probes). All reagents for PAGE were nuclease-free grade.

To test the stability of ODNs complexed with liposome, each ODN was at first mixed with cationic liposome reagent (LipofectAMINE PLUS). Thus, 2.5 μl of each ODN stock solution (1 μM) and the plus reagent (5 μl) were mixed in MilliQ water (52.5 μl) in a small sterile tube. After immediate mixing with a Vortex mixer and standing at room temperature for 15 min, the LipofectAMINE reagent (2 μl) in MilliQ water (50 μl) was added, and mixture was left at room temperature for 15 min. 5.9 μl of FBS was added (final 5%) to the transfection complex (112 μl), and the mixture was incubated at 37 °C.

For calculation of the half-life of each ODN in serum, all bands were analyzed with an imaging densitometer, GelDoc 1000 system (Bio-Rad).
with cationic liposome reagent (LipofectAMINE PLUS) prior to incubation. An easily detectable internal standard. The stability of ODNs composed of various ODNs. The 20-mer in the markers is present in a 3-fold molar excess over the other sequences to serve as an easily detectable internal standard. The stability of ODNs composed with liposome was also examined. Various ODNs were mixed with cationic liposome reagent (LipofectAMINE PLUS) prior to incubation with 5% FBS as described under “Experimental Procedures.” A and E, 5′-3′-Inverted T AS; B and F, 3′-inverted T AS; C and G, PS AS; D and H, 5′-3′-non-inverted T AS.

Toxicity of ODNs—ODNs (200 μM 5′-3′-inverted T AS or 50 μM PS AS) in 50 μl of 1.75% atelocollagen were subcutaneously injected into both lower flanks of 8-week-old male athymic nude mice. One and 7 days after injection, blood was taken from the infranuclear vein and sera were analyzed using HITACHI Clinical Analyzer 7170 by SRL, Inc. (Nagoya, Japan). Seven days after injection, mice were killed, and gross anatomy of organs was examined.

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

RESULTS

Effects of 5′-3′-Inverted T AS on CMT-93 Carcinoma Cells in Culture—We examined whether 5′-3′-inverted T AS can decrease MK secretion in CMT-93 rectal carcinoma cells. 5′-3′-Inverted T AS transfected with the aid of a cationic liposome reagent (LipofectAMINE PLUS) suppressed synthesis and secretion of MK (Fig. 2A, lane 1), whereas 5′-3′-inverted T SEN and 5′-3′-inverted T REV showed no effects (Fig. 2A, lanes 2 and 3). Densitometric analysis of the blots revealed that 5 μM 5′-3′-inverted T AS decreased MK production to 9% of that in controls (Fig. 2A, lane 8). We also observed that 3′-inverted T AS and PS AS exhibited almost identical effects after transfection (Fig. 2A, lanes 4 and 5). These inhibitory effects on MK production by 5′-3′-inverted T AS and 3′-inverted T AS were dose-dependent (Fig. 2B).

We also examined whether the nature of the complex formed between the cationic lipid delivery agent and ODNs were different depending on ODNs. When FITC-labeled ODNs were mixed with LipofectAMINE PLUS and observed by confocal microscopy, the diameter of the particles of the labeled complex was 278.5 ± 65.6 nm (n = 20) for PS AS, 293.5 ± 53.8 nm (n = 20) for PS SEN, 285.3 ± 48.4 nm (n = 20) for 5′-3′-inverted T AS, 310.4 ± 53.9 nm (n = 20) for 5′-3′-inverted T SEN, 298.6 ± 43.6 nm (n = 10) for 5′-3′-inverted T REV, and 274.5 ± 43.5 nm (n = 10) for 3′-inverted T AS. The results indicated that the size of the complex was not different between PS AS, 5′-3′-inverted T AS, 3′-inverted T AS, and other controls.

To measure the amount of ODNs bound to liposomes, we relied on the observation that ODNs complexed with liposomes stay within the dialysis tube after dialysis, whereas free ODNs are dialyzed out (34). Indeed, without LipofectAMINE reagents, only 2% of 32P-labeled ODNs stayed in the dialysis apparatus after the procedure described under “Experimental Procedures.” However, around 70–80% of 32P-labeled ODNs were in the dialysis apparatus in the presence of the LipofectAMINE reagents. Actual values were as follows: 82.2% for 5′-3′-inverted T AS, 73.0% for 3′-inverted T AS, and 72.7% for PS AS. Therefore, the efficiency of the complex formation was not significantly different between different ODNs. In the transfection complex the amount of ODN was 5 nmol, of which about 75% formed complex with liposomes, and the amount of lipids was 6.8 nmol. Thus, the ratio of ODNs to lipids in the liposome was about 1:1.8.

Cytotoxicity of 5′-3′-Inverted T ODNs and PS ODNs—When a high dose of PS SEN was added to cultures of CMT-93 cells, it showed noticeable cytotoxic effects on survival of the cells in a dose- and time-dependent manner (Fig. 3B). On the other hand, 5′-3′-inverted T SEN showed almost no cytotoxicity at least up to 48 h (Fig. 3A).

Cellular Uptake of ODNs—The cellular uptake and distribution of 5′-3′-inverted T SEN and PS SEN were examined by confocal microscopy. As shown in Fig. 4, both FITC-labeled 5′-3′-inverted T SEN and PS SEN showed similar cellular distributions, mainly in nuclei as stained by propidium iodide, indicating that both ODNs entered the cells and reached the inside of the cells upon cationic liposome-mediated transfection. FITC-labeled 5′-3′-inverted T AS, 5′-3′-inverted T REV, 3′-inverted T AS, and PS AS showed similar localization and
the case of 5'-3' antisense ODN did not change their effects on MK production in the difference of uptake affected the result. The presence or absence of FITC did not change their effects on MK production in the cell, excluding the possibility that the relative intensity in the cell, excluding the possibility that the relative difference of uptake affected the result. The presence or absence of FITC did not change their effects on MK production in the case of 5'-3' antisense T AS, 5'-3' inverted T SEN (open circles), PS AS, or PS AS (open triangles) was mixed with atelocollagen, and 50 μl of each mixture was injected into the tumor region, as indicated in the figure. Day 0 corresponds to 9 days after inoculation of cells, when tumor volume was ~60 mm^3. A, tumor growth curves. Tumor diameters were measured at regular intervals for up to 41 days with digital calipers, and tumor volume was calculated. Results represent the means ± S.E. (n = 6). #, p < 0.001 versus 5'-3' inverted T SEN and PS SEN. *, p < 0.01 versus 3'-inverted T AS and PS AS. Injection of PBS yielded results indistinguishable from those obtained upon injection of PS SEN (32). B, tumor weights. The mice were sacrificed 41 days postinjection, and all tumors were excised, and the tumor weights were determined. Results represent the means ± S.E. (n = 6). #, p < 0.001 versus 5'-3' inverted T SEN and PS SEN; *, p < 0.01 versus 3'-inverted T AS and PS AS.

Stability of 5'-3'-inverted T-modified AS in FBS—ODNs with the natural PO backbone are digested by nucleases in less than 5 min in vivo, making them unsuitable for therapeutic use (4, 5). PS-modified ODNs are considerably more stable in vivo (6, 37). Any modified ODN that would be useful as an antisense agent should show reasonable stability against nucleases as well as acceptable hybridization with the target mRNA. Thus, we studied the stability of ODNs in DMEM supplemented with 5% heat-inactivated FBS. Fig. 5 shows the stability of the ODNs under these conditions. 5'-3'-Non-inverted T AS was quickly digested by nucleases in serum, consistent with the reported data on PO-ODN (4). At 24 h, only a faint band of intact ODN was present (Fig. 5D). Unexpectedly, PS-modified ODN was not stable in serum, as indicated in Fig. 5C. In contrast to the above observations, 5'-3'-inverted T AS and 3'-inverted T AS were strongly resistant to nucleases in serum (Fig. 5, A and B). In the case of 5'-3' inverted T AS, even though intact 5'-3'-inverted T AS disappeared by 24 h, the produced fragment, probably 3'-inverted T AS, remained intact for up to 96 h. The half-lives of each ODN determined by densitometric analyses were as follows: 5'-3'-inverted T AS, 30 h; 3'-inverted T AS, 110 h; PS AS, 10 h; 5'-3'-non-inverted T AS, 5 h.

We also examined the stability of the complex of ODNs and LipofectAMINE PLUS to serum nucleases (Fig. 5). The complex formation to liposome generally increased the stability of ODNs, and more than half 5'-3'-inverted T AS and most 3'-inverted T AS remained as the original one even after 96 h. As compared with them, PS AS and non-inverted T AS were still much more unstable.

Growth Inhibitory Effects of ODNs on CMT-93 Cells—Transfection of 5'-3'-inverted T AS into CMT-93 cells inhibited their growth especially 3–5 days after addition (Fig. 6). However, 5'-3'-inverted T SEN showed no such effects (Fig. 6). We also observed a similar inhibitory effect of 3'-inverted T AS.
Treatment of Preformed Tumors by MK Antisense ODN—We then injected preformed tumors with 5′-3′-inverted T AS. Untreated CMT-93 cells were inoculated into nude mice as described previously (32). Nine days after inoculation when a palpable tumor was formed, 5′-3′-inverted T AS, 3′-inverted T AS, PS AS, 5′-3′-inverted T SEN, or PS SEN, which were premixed with atelocollagen, was injected into the tumor, and the injection was repeated every 14 days. 5′-3′-Inverted T AS markedly suppressed tumor growth as compared with 5′-3′-inverted T SEN and PS SEN ($p < 0.001$) (Fig. 7A). 3′-Inverted T AS also significantly suppressed tumor growth as compared with 5′-3′-inverted T SEN ($p < 0.001$). PS AS also suppressed tumor growth, as reported previously (Ref. 32, Fig. 7A). As tumor volume at the initial injection in the present experiment (Fig. 7A) was larger than that in the previous report, the effect of PS AS was slightly less as compared with that in the previous report (32). We found that the tumor-suppressive effect of 5′-3′-inverted T AS was greater than that of 3′-inverted T AS and PS AS ($p < 0.01$).

In the above experiment, tumor growth was monitored by determining tumor volume estimated with digital calipers. At the end of the study (41 days after initiation of tumor therapy), all animals were sacrificed, and tumor weight was determined. 5′-3′-Inverted T AS significantly suppressed the increase of tumor weight as compared with controls (Fig. 7B).

We used atelocollagen as the carrier of ODNs, because liposomes do not consistently give good delivery in vivo (38). Indeed, 5′-3′-inverted T AS showed much weaker effects when LipofectAMINE PLUS was used as a carrier (Fig. 8).

Injection of 5′-3′-inverted T AS or PS AS in atelocollagen at the dose used for tumor therapy did not show apparent toxicity to nude mice. Thus, serum levels of creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase were not different between control animals and those injected with the above ODNs in atelocollagen (data not shown). Gross anatomy of organs was also unaffected.

**DISCUSSION**

Antisense therapeutics using synthetic oligonucleotides are currently being evaluated in clinical trials for cancer, inflammation, and viral diseases (12). Most antisense ODNs under development for clinical application contain a PS backbone, whereas side effects and toxicities were reported to be induced by PS-modified ODNs (12). In primates, the primary acute side effects are associated with complement activation (13, 39) by PS-modified ODNs (12). In primates, the primary acute side effects are associated with complement activation (13, 39–41) and systemic effects due to accumulation of high concentrations of PS-modified ODNs in the kidneys (39, 42). In rodents, the primary side effect is immune stimulation characterized by lymphoid hyperplasia and mononuclear cell infiltrates in multiple tissues (14). At extraordinarily high doses (15–50 times the planned clinical doses), hepatocellular (>50 mg/kg) and renal tubular degeneration (>100 mg/kg) are evident in rodents (14, 15, 43, 44). In the liver, Kupffer cell hyperplasia and exacerbation of background foci of inflammatory cells were observed as typical effects induced by PS-modified ODNs (12), although the mechanism of the liver toxicity was not clear.

To overcome the toxic effects of PS-modified ODNs described above, 5′-3′-inverted T antisense ODNs were devised as new reagents to suppress gene expression, and their effectiveness was confirmed by suppression of MK synthesis, leading to suppression of growth of CMT-93 rectal carcinoma in nude mice. 5′-3′-Inverted T MK antisense ODN indeed exhibited less toxicity than the PS MK antisense ODN. Thus, we could administer larger amounts of 5′-3′-inverted T MK antisense ODN and obtain better therapeutic effects than with the use of PS MK antisense ODN.

In the presence of serum, 20-mer 5′-3′-inverted T AS was converted to a slightly shorter oligonucleotide, probably a 19-mer, and remained stable. On the other hand, the 19-mer 3′-inverted T AS was stable in serum. These results suggested that 5′-inverted T of 5′-3′-inverted T AS was susceptible to an exonuclease in the serum, whereas the 3′-inverted T was resistant to the nuclease. Consistent with these observations, 5′-3′-inverted T AS and 3′-inverted T AS exhibited similar growth inhibitory activity to CMT-93 rectal carcinoma cells. However, 5′-3′-inverted T AS exhibited stronger antitumor activity in nude mice than 3′-inverted T AS. Exonucleases in the tumor tissue probably degraded these AS in a manner different from that in serum.

Recently, various new strategies have been introduced to produce antisense DNA, e.g., replacement of the sugar-phosphate backbone to 2-aminoethyl glycine (16, 45, 46), replacement of PO linkages with amide linkages (18, 19), and morpholino-oligonucleotides (20). Comparative evaluation of the 5′-3′-inverted T AS and these new reagents should be performed in future studies.

Nucleotide-mediated therapies require effective delivery systems. Atelocollagen is produced by elimination of antigenic telopeptides attached to both ends of the collagen with pepsin (47). Thus, it is neither antigenic nor toxic in animals. In addition, atelocollagen is liquid at 4 °C and a gel at 37 °C. Satisfactory delivery of plasmid DNA (47) and PS-modified ODNs (32) via atelocollagen was recently achieved. The present study shows that atelocollagen is also suitable for delivering another DNA structure, 5′-3′-inverted T ODN, and supports the usefulness of atelocollagen in delivery of various DNA compounds.

The antisense strategy is a hopeful new approach to cure malignant tumors (48, 49). Molecules with anti-apoptotic activity are frequent targets of such antisense therapy (48). As MK is also known to have anti-apoptotic activity (50, 51) and is overexpressed in a number of carcinomas (25–30), antisense MK ODNs in the form of 5′-3′-inverted T or other forms are excellent candidates for testing for clinical effectiveness in cancer therapy.

**Acknowledgments**—We thank Michio Watanabe (Amersham Biosciences) and Yuka Ishida (ATTO Corp.) for helpful suggestions in PAGE of ODNs. We also thank Kanako Yuasa for excellent technical assistance.

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