Sp1 Decoy Transfected to Carcinoma Cells Suppresses the Expression of Vascular Endothelial Growth Factor, Transforming Growth Factor β1, and Tissue Factor and Also Cell Growth and Invasion Activities

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ABSTRACT

Vascular development is thought to be an important aspect in the growth and metastasis of solid tumors. Among the angiogenic factors produced by tumor cells, vascular endothelial growth factor is considered to be the most potent and pathologically important. The synthesis of this growth factor has been shown to be modulated through Sp1 function following stimulation by tumor necrosis factor α (TNF-α). Oligodeoxynucleotides (ODNs) were synthesized with either the consensus sequence for Sp1 binding (Sp1 decoy ODNs) or a mutated form of this sequence (mt-Sp1 decoy ODNs). Using the hemagglutinating virus of Japan (HVJ)-liposome method, we transferred these ODNs into cultured cancer cells (A549 and U251 cells). The TNF-α-mediated expression of both VEGF and transforming growth factor β1 and tissue factor (TF) by the cancer cells could be simultaneously suppressed to less than 30% by transfection of Sp1 decoy ODNs but not by mt-Sp1 decoy ODNs. In addition, in vitro invasiveness, synthesis of mRNA for urokinase-type plasminogen activator, and cell proliferation of both cell lines were also inhibited to 40% by the transfection of only Sp1 decoy ODNs. These results suggested that the Sp1 decoy strategy would be effective for regulating tumor growth by simultaneously reducing cancer cell (a) angiogenic growth factor expression, (b) proliferation, and (c) invasiveness.

INTRODUCTION

The progressive growth of solid tumors depends on vascularization from the surrounding stromal tissue into the tumor tissue (1–3). There are several reports on clinical trials of anticancer therapy by inhibition of angiogenesis with small molecules, such as linomide (4), TNP-470 (5), thalidomide (6), marimastat (7), suramin (8), angiostatin (9), and endostatin (10). In the angiogenic process, tumor cells are thought to play a critical role by producing and secreting a number of angiogenic factors (1, 2). Of these, VEGF is multipotent with marked effects on cell proliferation, differentiation, adhesion, and migration, as well as other activities (24). It is also reported to be regulated by Sp1 activation (25). Although the mechanism of the angiogenic effects of VEGF has not been clearly defined, TF stimulation of angiogenesis in vitro by local injection (26) and in vivo via the overexpression of TF is reported to be highly expressed in several cancer cells and enhances the metastatic potential of these cells (28, 29). In addition, TF is reported to affect tumor growth (30). Furthermore, a recent report indicated that VEGF synthesis is dependent on TF in response to FVIIa (31). This suggests that TF may influence tumor angiogenesis through regulation of VEGF synthesis. TF is reported to be transactivated by NF-κB, AP-1, and Sp1 under several stimuli. The TNF-α-induced transcription of the TF gene is thought to be regulated by Sp1 (32).

Simultaneous inhibition of the synthesis of these angiogenic factors and other proteins related to cell migration and proliferation, such as uPA, by altering Sp1 action via Sp1 decoy strategy, should dramatically suppress tumor growth. This study examined the inhibitory effects of a cis-trans element decoy containing Sp1 binding sites on the TNF-α-induced synthesis of VEGF by tumor cells. TGF-β1, produced by tumor-associated macrophages, through the action of transcription factor Sp1 (23).

In this study, we examined the inhibitory effects of a cis-trans element decoy containing Sp1 binding sites on the TNF-α-induced synthesis of VEGF by tumor cells. TGF-β1 is multipotent with marked effects on cell proliferation, differentiation, adhesion, and migration, as well as other activities (24). It is also reported to be regulated by Sp1 activation (25). Although the mechanism of the angiogenic effects of TGF-β1 is not completely clear, TGF-β1 stimulates angiogenesis in vivo by local injection (26) and in vitro via the stimulation of tube formation by cultured capillary endothelial cells (27). TF is a transmembrane glycoprotein that can be a potent cofactor of FVIIa. The TF/FVIIa complex initiates the coagulation cascade leading to thrombin formation. TF is thought to be a genuine receptor inducing an intracellular signaling response on binding to its specific ligand, FVII. TF is reported to be highly expressed in several cancer cells and enhances the metastatic potential of these cells (28, 29). In addition, TF is reported to affect tumor growth (30). Furthermore, a recent report indicated that VEGF synthesis is dependent on TF in response to FVIIa (31). This suggests that TF may influence tumor angiogenesis through regulation of VEGF synthesis. TF is reported to be transactivated by NF-κB, AP-1, and Sp1 under several stimuli. The TNF-α-induced transcription of the TF gene is thought to be regulated by Sp1 (32).

In this study, we examined the inhibitory effects of a cis-trans element decoy containing Sp1 binding sites on the TNF-α-induced synthesis of VEGF by tumor cells. TGF-β1 is multipotent with marked effects on cell proliferation, differentiation, adhesion, and migration, as well as other activities (24). It is also reported to be regulated by Sp1 activation (25). Although the mechanism of the angiogenic effects of TGF-β1 is not completely clear, TGF-β1 stimulates angiogenesis in vivo by local injection (26) and in vitro via the stimulation of tube formation by cultured capillary endothelial cells (27). TF is a transmembrane glycoprotein that can be a potent cofactor of FVIIa. The TF/FVIIa complex initiates the coagulation cascade leading to thrombin formation. TF is thought to be a genuine receptor inducing an intracellular signaling response on binding to its specific ligand, FVII. TF is reported to be highly expressed in several cancer cells and enhances the metastatic potential of these cells (28, 29). In addition, TF is reported to affect tumor growth (30). Furthermore, a recent report indicated that VEGF synthesis is dependent on TF in response to FVIIa (31). This suggests that TF may influence tumor angiogenesis through regulation of VEGF synthesis. TF is reported to be transactivated by NF-κB, AP-1, and Sp1 under several stimuli. The TNF-α-induced transcription of the TF gene is thought to be regulated by Sp1 (32).

MATERIALS AND METHODS

Cells. Human lung adenocarcinoma (A549) and human glioblastoma multiform (U251) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 10% FBS (Flow Laboratories, Stanmore, Australia), penicillin (100 units/ml), and streptomycin (100 mg/ml) in a humidified incubator (Multi-Gas incubator BL3200; Astec Co., Fukuoka, Japan) in 5% CO2 at 37°C.

Synthesis of Sp1 Decoy ODNs and Selection of Target Sequences. ODNs included the binding sequence (underlined) for transcriptional factor Sp1 centrally and dummy sequences upstream and downstream from the Sp1 binding sequence (33). Double-stranded ODNs were prepared by incubation at

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor α; ODN, oligodeoxynucleotide; TF, tissue factor; FVII, factor VII; uPA, urokinase-type plasminogen activator; FBS, fetal bovine serum; HVJ, hemagglutinating virus of Japan; EMSA, electrophoretic mobility shift assay; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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90°C for 5 min and were designated “Sp1 decoy ODNs.” Mutated Sp1 decoy ODNs (designated “mt-Sp1 decoy ODNs”) were mutat at two positions (shown as lowercase letters) of the Sp1 binding sequence found in the Sp1 decoy ODNs. Inspection of the resulting nucleotide sequences showed no sequence homologies to other known transcription factors using databases on transcriptional regulation (34): Sp1 decoy ODNs, 5′-ATTACGGCGCG- 

GGGGCTAC-3′ and 3′-GTAGGCCCGCCGGCTTAAT-5′; mt-Sp1 decoy ODNs, 5′-ATTACGGGA GTAGGGCTAC-3′ and 3′-GTAGCCCT a t 

CTACGGGTAT-5′.

To enhance the transport of the transfected decoy ODNs to the nucleus, protein-DNA complexes with high mobility group proteins HMG-1 and -2 were prepared before transfection by incubating the ODNs at 20°C for 20 min with high mobility group proteins HMG-1 and -2.

HVJ-Liposome Transfection. In this study, we transferred ODNs into cancer cells using the HVJ-liposome method (35). Briefly, phosphatidylserine (sodium salt), phosphatidylcholine, and cholesterol (all purchased from Sigma Chemical Company, St. Louis, MO) were mixed in a weight ratio of 1:4:8.2 in 3.9 ml of tetrahydrofuran. The lipid mixture (10 mg) was deposited on the HVJ-liposome solution in balanced salt solution (10 mM Tris-HCl, 137 mM NaCl, 5.4 mM KCl; 100 HVJ-liposome particles/cell), containing either decoy ODNs or 3 µg of nuclear extracts. The mixture was incubated at 4°C for 10 min to adhere HVJ to liposomes. The mixture was incubated with gentle shaking at 37°C for 60 min to fuse liposome membrane and HVJ. Free HVJ was removed from HVJ-liposomes by sucrose density gradient centrifugation at 60,000 × g at 4°C for 3 h.

The HVJ-liposomes-containing Sp1 or mt-Sp1 decoy ODNs were stored at 4°C and used for the following transfection study. HVJ-liposomes prepared without ODNs (empty HVJ vehicles) were used as a control.

Gene Transfer. A549 and U251 cells were grown to 50% confluency. The 10% FBS-supplemented RPMI 1640 was replaced with FBS-free RPMI 1640 and was harvested 12 h later. The serum-free medium was exchanged for HVJ-liposome solution in balanced salt solution (10 mM Tris-HCl, 137 mM NaCl, 5.4 mM KCl; 100 HVJ-liposome particles/cell), containing either decoy ODNs or no ODNs. Transfer of the ODNs was allowed to occur by incubation at 37°C for 12 h. Cells were then washed five times with PBS to completely remove any HVJ-liposome that had not been transferred. Transfected cells were immediately used for the following experiments.

Flow Cytometry. To detect ODNs located within cultured cells after transfection, Sp1 and mt-Sp1 decoy ODNs were labeled at the 5′ end with FITC (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) and T4 polynucleotide kinase (Toyobo Co. Ltd., Osaka, Japan), respectively. Flow cytometry was used to observe A549 and U251 cells 3 h after FITC-labeled Sp1 decoy ODNs’ transfection. Cells were trypsinized, washed three times with PBS, and analyzed on a FACScan II (Becton Dickinson, Mountain View, CA), gating on live cells. Fluorescence was monitored at 488 nm with a 525-nm band pass filter. A549 and U251 cells without transfection of FITC-labeled decoy ODNs were used as a control.

Fluorescence Microscopic Examination. The intracellular location of decoy ODNs in the cultured cells transfected with HVJ-liposome was determined by fluorescent microscopy of cells transfected with FITC-labeled decoy ODNs. A549 and U251 cells were seeded on chamber slides and incubated with 10% FBS-supplemented medium for 12 h to allow the cells to adhere. Three and 6 h after initiation of the transfection protocol described above, cells were washed with PBS and subjected to microscopic examination. A549 and U251 cells without transfection of FITC-labeled decoy ODNs were the controls.

EMISAs. After replacing conditioned media, supplemented with serum, with serum-free media containing TNF-α (100 ng/ml; R&D Systems, Minneapolis, MN), nuclear proteins were isolated from cancer cells before and 15, 30, 60, and 120 min after TNF-α treatment (36). Twenty µg of nuclear extracts were subjected to EMSA as reported previously (37). Sp1 decoy ODNs or mt-Sp1 decoy ODNs (0.35 pmol/ml) were end-radiolabeled using T4 polynucleotide kinase, [γ-32P]dATP, and commercially available buffer (Life Technologies, Inc., Rockville, MD) for a 30-min incubation at 37°C in a total volume of 10 µl. The reaction was stopped with EDTA. Ten µg of nuclear extract and 2.0 µg of poly(deoxyinosinic-deoxyctydilic acid) were incubated with the radiolabeled Sp1 decoy ODNs or mt-Sp1 decoy ODNs in a total volume of 15 µl. ODN-protein complexes were analyzed by electrophoresis through 5% polyacrylamide gels in 22.3 mM Tris, 22.3 mM boric acid, and 0.5 mM EDTA. Visualization was performed using a BAS 2000 Bioimage Analyzer (Fuji Photo Film Co., Tokyo, Japan).  

**Northern Blot Analysis.** Radiolabeled cDNA probes (VEGF 165, TGF-β1, TF, bFGF, and uPA) were prepared according to the manufacturer’s instructions with [α-32P]dCTP (Amersham) and a DNA Labeling Kit purchased from Pharmacia Biotech (dCTP).

Total RNA was examined to determine the effects of ODNs on TNF-α-mediated expression of the VEGF, TGF-β1, TF, bFGF, and uPA genes. A549 and U251 cells with or without transfection of ODNs (Sp1 decoy ODNs or mt-Sp1 decoy ODNs) were treated with TNF-α (100 ng/ml) for 12 h. Total RNA was isolated from the cells by the guanidine isothiocyanate extraction method described previously (38). Twenty µg of total RNA were electrophoresed on 1.2% agarose/3% formaldehyde DNA gels and transferred onto N nylon membranes (Amersham Life Sciences, Amersham, United Kingdom). The membranes were prehybridized in 1× SSC, 0.5 M NaHPO4 (pH 7.2), and 7% SDS at 52°C for 2 h. Hybridization was performed with purified, radiolabeled VEGF, TGF-β1, TF, bFGF, or uPA cDNA in the above solution at 52°C for 16 h. The membrane was initially washed in 0.1× SSC,0.1% SDS at 52°C. Visualization was performed using the BAS 2000. The mRNA expression was quantitatively evaluated using a densitometer. Applied total RNA was routinely standardized with either human GAPDH mRNA expression or ethidium bromide-stained rRNA.

**ELISA.** The presence of VEGF and TGF-β1 proteins secreted by A549 and U251 cells into conditioned medium was determined with a human Quantikine Kit from R&D Systems. VEGF or TGF-β1 concentrations were quantitatively determined according to the manufacturer’s instructions with minor modifications relative to standard curves derived from diluted recombinant human VEGF165 or TGF-β1 proteins. ELISA values were read using a microplate reader (Immuno-mini, NJ-2300; Inter Med, Tokyo, Japan).

**Cell Growth.** A549 and U251 cells were plated onto tissue culture wells at 1 × 104 cells/2.1 cm2, cultured with RPMI 1640 containing 10% FBS for 12 h to allow adherence. This was followed by incubation with serum-free medium for 12 h. After transfection by the HVJ-liposome method, the culture medium was replaced with RPMI 1640 containing 10% FBS, renewed every 2 days. Cell cultures were washed twice with PBS and removed from the tissue culture wells by trypsinization (0.25% in PBS) at 3, 6, and 9 days. Cell numbers were determined using a hemocytometer. Both of the cell lines with or without transfection of empty HVJ-liposome were used as controls. Each experiment was performed in triplicate. The results are given as mean cell numbers ± SD/8 dish.

**In Vitro Migration Assay.** The Boyden chamber procedure was used as described elsewhere (39) to evaluate cell migration activity in a 24-microwell chemotaxis chamber (Falcon). The upper and lower wells were separated by a polycarbonate filter (8 µm pore size), coated with type I collagen and fibronectin. The lower wells contained RPMI 1640 with 10% FBS. Cell suspension (1.2 × 105 cells in 50 µl of serum-free medium) of A549 and U251 cells, transfected with Sp1 decoy ODNs or mt-Sp1 decoy ODNs, or treated with empty HVJ-liposomes, was added to the upper wells. The chamber was incubated at 37°C for 6 h. The filters were then removed and fixed in methanol overnight. Nonmigrating cells on the upper surface of the filters were removed with a cotton swab. Cells were stained with Giemsa and counted using a microscope at ×40 in 10 random fields per each well. Migration was assayed by measuring the number of cells moving across the filter. Each experiment was performed in triplicate. The number of cells that migrated is given as the mean ± SD. The number of cells that migrated without any treatment was also determined.

**Statistical Analysis.** Results are expressed as the mean ± SD. Statistical comparison of several means was carried out by an ANOVA, and comparison of two means was done with Student’s t test. All of the Ps were analyzed on two sides.
by TNF-α of these mRNA species were increased in a dose-dependent manner after stimulation by 100 ng/ml of TNF-α alone (Fig. 2).

The cytotoxic effect on cultured cells was observed with this TNF-α treatment for 12 h with the indicated concentration of TNF-α (0, 10, 50, and 100 ng/ml) total RNA was isolated from cells. For each lane, 20 μg/ml of total RNA was electrophoresed through 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with radiolabeled VEGF cDNA probe. Visualization was performed by an image analyzer. Human GAPDH was the internal control.

The effects of the transfection of decoy ODNs transfection on mRNA synthesis of TNF-α-induced angiogenic factors. Total RNA was isolated from A549 and U251 cells cultured for 12 h in the presence of TNF-α (100 ng/ml) after transfection of Sp1 decoy ODNs (Decoy), mt-Sp1 decoy ODNs (mt-Decoy) and treatment with empty HVJ-liposome (Empty). Both of the cell lines without transfection were cultured for 12 h in the presence or absence of TNF-α (100 ng/ml). Total RNA (20 μg/ml) was electrophoresed in 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with radiolabeled cDNA probes. Visualization was performed by an image analyzer. The mRNA expression was quantified using a densitometer. The number with each lane, the relative intensity. Applied total RNA was standardized by ethidium bromide staining of rRNA.

RESULTS

The Effects of TNF-α Treatment on VEGF, TGF-β1, and TF Expression. The effect of TNF-α treatment on the expression of VEGF, TGF-β1, and TF was examined in A549 and U251 cells. All of these mRNA species were increased in a dose-dependent manner by TNF-α treatment (0, 10, 50, and 100 ng/ml) for 12 h. The amounts of VEGF, TGF-β1, and TF mRNA were most prominent in both of the cell lines after stimulation by 100 ng/ml of TNF-α. The increases were about 4- (VEGF and TGF-β1) and 2-fold (TF; Figs. 1 and 2). No cytotoxic effect on cultured cells was observed with this TNF-α dose. Subsequent experiments used 100 ng/ml TNF-α. Treatment with TNF-α (100 ng/ml) had no effect on bFGF mRNA synthesis in A549 and U251 cells compared with treatment with serum-free medium alone (Fig. 2).

Transactivation of Sp1 in A549 and U251 Cells Treated with TNF-α. To demonstrate the transactivation of Sp1 in A549 and U251 cells after TNF-α treatment, EMSA was performed using radiolabeled Sp1 decoy ODNs or mt-Sp1 decoy ODNs as a probe. The binding of Sp1 decoy ODNs to nuclear protein extracted from either A549 or U251 cells was observed within 15 min after stimulation with TNF-α (100 ng/ml) and continued for at least 120 min (Fig. 3). No complex formation with radiolabeled mt-Sp1 decoy ODNs was detected. These results indicate not only that TNF-α treatment induces the transactivation of Sp1 in the cancer cells examined, but also that the sequences of the Sp1 decoy ODNs bind the activated Sp1 protein, whereas those of the mt-Sp1 decoy ODNs do not.

In Vitro Transfection Efficiency and Localization of FITC-labeled Decoy ODNs in Cancer Cells. To examine the transfer efficiency of decoy ODNs into A549 and U251 cells using the HVJ-liposome vector, we performed flow cytometry on the cultured cells 3 h after transfection of FITC-labeled Sp1 decoy ODNs. Although the efficiency of transfection varied slightly between attempts, more than 80% of both of the cell lines was routinely transfected with FITC-labeled decoy ODNs (Fig. 4).

The intracellular localization of FITC-labeled decoy ODNs in A549 and U251 cells was examined by fluorescence microscopy 3 and 6 h after transfection of Sp1 decoy ODNs. Three h after transfection, FITC-labeled decoy ODNs were mainly localized in the cell cytoplasm (Fig. 5a). Six h after transfection, FITC-labeled decoy ODNs were localized in the nucleus (Fig. 5b).

The Effect of Sp1 Decoy ODNs Transfection on TNF-induced Expression of VEGF, TGF-β1, and TF mRNAs. Total RNA from A549 and U251 cells transfected with either Sp1 decoy ODNs or mt-Sp1 decoy ODNs were stimulated with TNF-α (100 ng/ml) for 12 h was examined for the amount of various mRNA species. Cells treated with empty HVJ-liposomes were used for the mock treatment.

Fig. 1. A Northern blot analysis of the A549 and U251 cells for VEGF mRNA. After treatment for 12 h with the indicated concentration of TNF-α (0, 10, 50, and 100 ng/ml) total RNA was isolated from cells. For each lane, 20 μg/ml of total RNA was electrophoresed through 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with radiolabeled VEGF cDNA probe. Visualization was performed by an image analyzer. Human GAPDH was the internal control.

Fig. 2. Effects of the transfection of decoy ODNs transfection on mRNA synthesis of TNF-α-induced angiogenic factors. Total RNA was isolated from A549 and U251 cells cultured for 12 h in the presence of TNF-α (100 ng/ml) after transfection of Sp1 decoy ODNs (Decoy), mt-Sp1 decoy ODNs (mt-Decoy) and treatment with empty HVJ-liposome (Empty). Both of the cell lines without transfection were cultured for 12 h in the presence or absence of TNF-α (100 ng/ml). Total RNA (20 μg/ml) was electrophoresed in 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with radiolabeled cDNA probes. Visualization was performed by an image analyzer. The mRNA expression was quantified using a densitometer. The number with each lane, the relative intensity. Applied total RNA was standardized by ethidium bromide staining of rRNA.

Fig. 3. The effects of TNF-α stimulation on Sp1 activity in A549 and U251 cells. Nuclear extracts were prepared from A549 and U251 cells incubated for the indicated times in the presence of TNF-α (100 ng/ml). Nuclear extracts were incubated with 32P-labeled Sp1 decoy ODNs or mt-Sp1 decoy ODNs and were analyzed by EMSA. Visualization was performed by an image analyzer. Solid arrowhead, the position of the specific complex of Sp1 and decoy ODNs. Open arrowhead, the position of the free decoy ODNs.

Fig. 4. In vitro expression of FITC-labeled decoy ODNs in A549 and U251 cells by HVJ-liposome method. Three h after transfection of FITC-labeled mt-Sp1 decoy ODNs to A549 and U251 cells by the HVJ-liposome method, both cells were subjected to flow cytometry to examine the transfection efficiency. Cells not undergoing transfection were used as controls.
control. As shown in Fig. 2, the treatment of both cell lines with Sp1 decoy ODNs reduced the TNF-α-induced mRNAs expression, i.e., 9% (A549) and 30% (U251) for VEGF mRNA, 23% (A549) and 8% (U251) for TGF-β1 mRNA, and 14% (A549) and under constitutive level (U251) in TF mRNA. In contrast, transfection of mt-Sp1 decoy ODNs or treatment with empty HVJ-liposomes had no effect on the TNF-α-induced synthesis of VEGF, TGF-β1, or TF mRNA in either A549 or U251 cells.

Treatment of A549 and U251 cells with TNF-α (100 ng/ml) for 12 h had no effect on the synthesis of bFGF mRNA relative to FBS-free medium alone. Treatment of A549 and U251 cells with either ODNs or with empty HVJ-liposomes had no effect on bFGF mRNA synthesis regardless of treatment with TNF-α (100 ng/ml).

The Effects of Transfection with Sp1 Decoy ODNs on TNF-induced VEGF and TGF-β1 Protein Production. To confirm that the effects of Sp1 decoy ODNs transfection on the synthesis of mRNA, described above, resulted in an effect on secretion of VEGF and TGF-β1 proteins by A549 and U251 cells into the conditioned media, the concentrations of VEGF and TGF-β1 proteins in conditioned media were determined with ELISA assays. As shown in Fig. 6, A and B, the secretion of these proteins was enhanced 3-fold with TNF-α stimulation (100 ng/ml). This TNF-α-induced secretion of both proteins was reduced to the respective constitutive level by transfection with Sp1 decoy ODNs. Neither transfection with mt-Sp1 decoy ODNs nor treatment with empty HVJ-liposomes had any effect on the secretion of VEGF and TGF-β1 proteins by A549 and U251 cells.

The Effects of Transfection of Sp1 Decoy ODNs on Cell Growth and Migration Activities. A549 and U251 cells underwent approximately four (A549) and three (U251) cell divisions over a 9-day period when maintained in RPMI 1640 containing FBS (Fig. 7, A and B). In contrast, transfection of Sp1 decoy ODNs reduced the growth of A549 and U251 cells on day 9, to 40 and 46%, respectively, relative to cells without any treatment. This inhibitory effect of Sp1 decoy ODNs on the growth of A549 and U251 cells is probably a consequence of reduction of Sp1 protein binding to the Sp1 consensus sequence in the promoter regions of the genes related to serum-dependent cancer cell growth. This interpretation is buttressed by the observation that neither mt-Sp1 decoy ODNs transfection nor empty HVJ-liposome treatment affected proliferation of either cell type.

Whether the Sp1 decoy ODNs transfection would influence the in vitro invasion activity of A549 and U251 cells through type I collagen and fibronectin in the presence of 10% FBS is assessed in Fig. 8. The invasiveness of A549 and U251 cells was reduced to about one-half by treatment with Sp1 decoy ODNs, as compared with no treatment. In contrast, either A549 and U251 cells transfection with mt-Sp1 decoy ODNs or treatment with empty HVJ-liposome had no effect on the invasiveness of the cells. The effect of Sp1 decoy ODNs transfection on TNF-α-induced uPA synthesis in A549 and U251 cells was evaluated by analysis of mRNA expression. Fig. 9 shows that treatment of A549 and U251 cells with Sp1 decoy ODNs reduced uPA mRNA expression. In contrast, transfection with mt-Sp1 decoy ODNs or treatment with empty HVJ-liposome had no effect on uPA mRNA synthesis in either A549 or U251 cells.

DISCUSSION

The data presented in this report clearly indicate that transfection of the Sp1 decoy ODNs into A549 and U251 cells, using the HVJ-liposome method, suppresses TNF-α-induced expression of the VEGF, TGF-β1, and TF genes. A comparable reduction in secreted VEGF and TGF-β1 protein by these cells in response to stimulation with TNF-α is presented. Transfection of both A549 and U251 cells by the Sp1 decoy ODNs also suppresses the invasion activities of these cells. Therefore, a possible advantage of the Sp1 decoy strategy is the simultaneous suppression of...
cells. This indicates that the Sp1 decoy ODNs used in this study bound activated Sp1, and mt-Sp1 decoy ODNs could be used as a negative control. Transfection of Sp1 decoy ODNs reduces the synthesis of TNF-α-induced VEGF, TGF-β1, and TF mRNA in both A549 and U251 cells by 10–30%, thereby reducing the levels of these mRNAs to approximately the constitutive levels of expression. These effects of Sp1 decoy ODNs are considered specific for Sp1-mediated transcription for three reasons. First, treatment with either mt-Sp1 decoy ODNs or empty HVJ-liposome had no effect. Second, transcription of the hFGF gene, thought to be independent of Sp1 function, was not influenced by either TNF-α treatment or transfection with either Sp1 decoy ODNs or mt-Sp1 decoy ODNs. Third, VEGF is believed to be up-regulated by hypoxia, via activation of transcription factors HIF-1 and AP-1. We confirmed induction of VEGF gene expression in both A549 and U251 cells under hypoxic conditions (data not shown). Both cell lines showed no alteration of hypoxia-induced VEGF synthesis with Sp1 decoy ODNs transfection (data not shown).

Both cell proliferation and the invasiveness of A549 and U251 cells were apparently reduced by transfection with Sp1 decoy ODNs. Neither transfection with mt-Sp1 decoy ODNs nor empty HVJ-liposome treatment showed any effect. Cancer cell invasiveness is thought to be regulated by many factors, such as matrix metalloproteases, uPA, cell-matrix interaction, and other factors (38). To clarify inhibitory mecha-

the transcription of several factors, including angiogenic molecules, through modification of Sp1 function.

Although there are many angiogenic factors related to tumor angiogenesis, VEGF is thought to be the most important growth factor in the vascularization of the adjacent stromal tissue, especially for solid tumors. Previous *in vitro* and *in vivo* studies of the inhibition of VEGF function used VEGF antisense mRNA (12–14), neutralizing antibodies against VEGF (15, 16), a soluble isoform of the VEGF receptor (17), and a dominant-negative isoform of the VEGF receptor (18). In this study, we tested a new “decoy” strategy as a specific gene therapy for cancers involving angiogenic suppression. The decoy strategy was originally reported by Bielinska *et al.* (20) and by Morishita *et al.* (21) who applied NF-κB decoy ODNs to suppress the acute inflammatory process in myocardial ischemia and reperfusion injury. VEGF synthesis is regulated by the transactivation of transcription factors such as AP-1, hypoxia-inducible factor-1 (HIF-1), and Sp1 (22). To regulate TNF-α-induced synthesis of VEGF, we selected a decoy system targeted to inhibit Sp1 function for several reasons: (a) inflammatory cytokines may play important roles in tumor progression via transcription factors including Sp1; (b) TNF-α can participate in tumor angiogenesis (23); and (c) synthesis of TNF-α itself is reported to depend on Sp1 activation (41).

Sp1 decoy ODNs, but not mt-Sp1 decoy ODNs, showed a mobility shift in the presence of nuclear extract prepared from TNF-α-treated

![Fig. 7. Effects of transfection of Sp1 decoy ODNs on A549 (A) and U251 (B) cell growth in 10% FBS-supplemented medium. A549 and U251 cells were plated at a density of 1 × 10⁵ cells/2.1 cm² well in serum-supplemented medium (10% FBS). Twelve h later, the cells were washed three times with PBS, had to serum-free medium, and incubated for 12 h. Then, transfection of Sp1 decoy ODNs or mt-Sp1 decoy ODNs was performed. Cells treated with empty HVJ liposome were also prepared. After transfection, serum-supple-
mented medium (10%) was added, and this was considered day 0. At the indicated number of days, the cells were washed two times with PBS, trypsinized, and counted with a hemocytometer. Cells not subjected to transfection were controls. The data represent the average of three separate experiments.

![Fig. 8. Effects of transfection of Sp1 decoy ODNs on A549 and U251 cells on invasion by 10% FBS-supplemented medium. A549 (□) and U251 (□) cells were transfected with Sp1 decoy ODNs (Decoy) or mt-Sp1 decoy ODNs (mt-Decoy). Cell lines from A549 and U251 treated with empty HVJ-liposome (Empty HVJ-lipo) were also prepared. All of these cell lines were suspended at a density of 1.2 × 10⁶ cells in 50 ml of serum-free medium and seeded on the upper surface of the filter of the chemotaxis chamber. The filter was precoated with type I collagen and fibronectin. Serum-supplemented medium (10%) was added to the lower wells and incubated at 37°C for 6 h. The number of cells on the lower side of the filter was counted. In addition, cell lines not subject to transfection were also examined in the absence or presence of FBS (10%). The data represent the number of cell lines of each experimental procedure was performed in triplicate. The means were compared statistically by an ANOVA, and pairs of means were compared with Student’s unpaired *t* test. All of the *Ps* were analyzed on two sides.

![Fig. 9. Effects of transfection of Sp1 decoy ODNs into A549 and U251 cells on invasion by 10% FBS-supplemented medium. A549 (□) and U251 (□) cells were transfected with Sp1 decoy ODNs (Decoy) or mt-Sp1 decoy ODNs (mt-Decoy). Cell lines from A549 and U251 treated with empty HVJ-liposome (Empty HVJ-lipo) were also prepared. All of these cell lines were treated with TNF-α (100 ng/ml) without transfection, and total RNA was examined. Total RNA was isolated from A549 and U251 cells cultured for 12 h in the presence of TNF-α (100 ng/ml) after transfection of Sp1 decoy ODNs (Decoy), mt-Sp1 decoy ODNs (mt-Decoy), and treatment with empty HVJ-liposome (Empty). In addition, both cell lines were cultured for 12 h in the presence or absence of TNF-α (100 ng/ml) without transfection, and total RNA was examined. Applied total RNA was standardized by human GAPDH mRNA expression.](https://cancerres.aacrjournals.org/article-pdf/60/20/6535/65506760.pdf)
nism of FBS-induced invasion, which the Sp1 decoy ODNs influences, we examined the TNF-α-induced synthesis of uPA mRNA. Transfection with Sp1 decoy ODNs inhibited TNF-α-induced synthesis of uPA mRNA in both cell lines. This is consistent with a previous report showing Sp1 binding site(s) in the promoter regions of the uPA gene (42). Although FBS contains many factors that stimulate cancer cell invasiveness, the inhibition of uPA synthesis by transfection with Sp1 decoy ODNs may be effective as a suppressor of invasiveness. As described above, the decoy strategy against Sp1 could theoretically provide a therapeutic method for inducing “tumor dormancy,” as proposed by Holmgren et al. (43), through the inhibition of both tumor angiogenesis and tumor cell growth and invasiveness. However, the strategy of treatment using decoy ODNs may possess both favorable and unfavorable effects in vivo. Therefore, a number of issues concerning the in vivo therapeutic potential of Sp1 decoy ODNs on tumor growth, such as transfer efficiency, duration of intranuclear retention of decoy ODNs in tumor tissue and side effects (both local and systemic), remain to be examined. In addition, Sp1 is considered a representative transcription factor, comparable with AP-1, NF-κB, and Egr-1 and -2, which participate in the induction and regulation of the inflammation-repair process. Therefore, the Sp1 decoy strategy may also be clinically pertinent in the treatment of inflammatory angiogenic diseases, including rheumatoid arthritis, atherosclerosis, and proliferative retinopathies.

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REFERENCES

Sp1 Decoy Transfected to Carcinoma Cells Suppresses the Expression of Vascular Endothelial Growth Factor, Transforming Growth Factor β₁, and Tissue Factor and Also Cell Growth and Invasion Activities

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