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Contiguous Four-guanosine Sequence in c-myc Antisense Phosphorothioate Oligonucleotides Inhibits Cell Growth on Human Lung Cancer Cells: Possible Involvement of Cell Adhesion Inhibition

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A contiguous four-guanosine (4G) sequence in c-myc antisense phosphorothioate oligonucleotides caused an antiproliferative effect in smooth muscle cells. To investigate the antiproliferative effect of c-myc antisense oligonucleotides on human lung cancer cell lines, we synthesized oligonucleotides of various lengths and sequences, focusing on the contiguous four-guanosine (4G) sequence. While a c-myc antisense oligonucleotide (20AS1 (4G)) targeted to the translation initiation codon of c-*myc* mRNA inhibited cell growth of A549 cells by 69% at 10 μM, a scrambled oligonucleotide (20SCR1 (4G)) containing the contiguous four-guanosine (4G) sequence also inhibited cell growth by 72% at the same dose. Although treatment with either 20AS1 (4G) or 20SCR1 (4G) inhibited cell adhesion by 70% at 10 μM, expression of c-myc protein was significantly suppressed only by 20AS1 (4G) (62%), and was only weakly inhibited by 20SCR1 (4G) (32%). Furthermore, a small cell lung carcinoma cell line, Lu65, which can grow in suspension form, was highly resistant to 20AS1 (4G) treatment ($IC_{50} > 20$ μM). These results suggest that the cell growth inhibition by c-myc antisense oligonucleotides containing the contiguous four-guanosine (4G) sequence was possibly correlated with inhibition of cell adhesion, but not with inhibition of c-myc protein expression, via a sequence-specific non-antisense mechanism.

Key words: c-myc antisense oligonucleotide — Contiguous four-guanosine (4G) sequence — Adhesion inhibition

Since the first successful inhibition of gene expression by an antisense oligonucleotide in 1978,[1] the potential usefulness of antisense oligonucleotides to inhibit expression of various genes has been widely documented.[2] Modified phosphorothioate oligonucleotides which retain the property of aqueous solubility and Watson-Crick base pair hybridization, but which are also nuclease-resistant, have been widely used to inhibit the synthesis of cellular or viral proteins *in vitro* and *in vivo*.[3,4] These compounds are potential therapeutic agents. Extensive pharmacological studies of phosphorothioate oligonucleotides have been performed *in vitro* and *in vivo* to develop clinically useful drugs.[5,6] Despite these successes, the mechanisms of action of phosphorothioate oligonucleotides remain speculative, and antisense mechanisms as well as a variety of non-antisense mechanisms have been proposed.[7] For example, binding of phosphorothioate oligonucleotides to proteins such as CD4, gp120, and SP1 has been documented.[8–10]

The c-*myc* proto-oncogene, which has essential roles in cell proliferation, malignant transformation, and apoptosis, is a potential target for antisense oligonucleotide treatment. Antisense phosphorothioate oligonucleotides targeted to the translation initiation codon of c-*myc* mRNA have been reported consistently to inhibit cell growth of a variety of cell types, including both normal and tumor cells.[11–15] A recent report demonstrated that a c-myc antisense oligonucleotide containing a contiguous four-guanosine (4G) sequence (targeted to the translation initiation codon) inhibited the proliferation of smooth muscle cell by a non-antisense mechanism.[16]

To develop antisense therapy for human lung cancer, we have chosen the c-*myc* proto-oncogene, which is deregulated in both small and non-small cell lung cancers,[17] as a target. We studied the biological effects of c-myc antisense phosphorothioate oligonucleotides on human lung cancer cells *in vitro*, and found that they inhibited cell growth in a sequence-non-specific manner, and the contiguous four-guanosine (4G) sequence was responsible for this growth inhibition. Furthermore, the adhesion-inhibitory effect of c-myc antisense oligonucleotides was also sequence-non-specific, and might be correlated with the growth inhibition of human lung cancer cells.

MATERIALS AND METHODS

Cell culture and cell growth inhibition assay Human lung adenocarcinomas (A549, RERELCMS), a human lung squamous cell carcinoma (Sq-19), and human lung small cell carcinomas (SBC-3, Lu65) were obtained from

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the Japanese Cancer Research Resources Bank. A human lung squamous cell carcinoma HS-24 was kindly provided by Dr. Muley (Heidelberg, Germany). A549 and RERFLCMS cells were cultured in EME medium with 10% fetal bovine serum (FBS), and Sq-19, HS-24, Lu65, and SBC-3 in RPMI-1640 medium with 10% FBS. Cell suspensions (1×10^5/2 ml) were mixed with the oligonucleotides at various concentrations (from 0.1 μM to 10 μM), and cultured in 6-well plates. Viable cells were counted every 24 h by the trypan blue dye exclusion method for 5 days. Cell viability was expressed as a percentage of that of untreated control cells taken as 100%.

Oligonucleotide synthesis Modified phosphorothioate oligonucleotides were synthesized by an Applied Biosystems 380B DNA synthesizer as previously described.[18] The oligonucleotides were purified by reverse-phase, high-performance liquid chromatography, and resuspended in distilled water. The sequences of oligonucleotides used are listed in Table I. The c-myc antisense oligonucleotide 20AS1 (4G) was targeted to the translation initiation codon of c-*myc* mRNA (Table I).

Western blot analysis of c-myc protein After a 24-h treatment with an oligonucleotide, A549 cells were lysed directly in Laemmli's buffer[19] with 5% mercaptoethanol. The samples were loaded onto 10% polyacrylamide/SDS gel, electrophoresed at 100 V for 2 h, and then transferred to a nylon membrane by using a Semi-Dry Transfer Cell (BIO-RAD, Richmond, CA). The membrane was incubated with anti c-myc monoclonal antibody (Ab-1, Oncogene Science, Uniondale, NY) for 2 h at room temperature, and then with alkaline phosphatase-conjugated anti mouse IgG antibody. Color was developed by use of the Proto Blot System (Promega, Madison, WI). The intensity of the c-myc protein band was quantitated by densitometry.

Northern blot analysis of c-*myc* mRNA Total RNA (20 μg) isolated with Isogene (Wako, Osaka) was denatured, fractionated in a 1.2% agarose gel containing formaldehyde (2.2 M), and transferred to a nylon membrane. The c-*myc* cDNA fragment (0.6 kb) was labeled with [α-^{32}P]dCTP by the use of a random primer labeling kit (Takara, Osaka). Hybridization was carried out with "Quick hyb" buffer (Stratagene, La Jolla, CA) at 68°C for 2 h. The membrane was also rehybridized with radiolabeled chicken *β-actin* cDNA probe (1.4 kb) as an internal control. The radioactivity of each band was determined using a Bio Imaging Analyzer, BAS-2000 (Fuji, Tokyo).

Cell adhesion studies Cell suspension (5×10^4/ml) was gently mixed with oligonucleotides at 1 μM and 10 μM, and then plated (5×10^3 cells/well) in 96-well plastic plates. The plates were incubated for 15 h, then nonadherent cells were removed by gentle pipeting and rinsing with a multi channel pipette. Cell numbers were quantitated by MTT assay of triplicate wells.

RESULTS

The contiguous four-guanosine (4G) sequence is responsible for cell growth inhibition of A549 cells A549 cells treated with a 20-mer antisense phosphorothioate oligonucleotide (20AS1 (4G)), targeted to the translation initiation codon of c-*myc* mRNA, showed marked cell growth inhibition in a dose-dependent manner (Fig.1, column 2), while a 20-mer sense oligonucleotide (20S) inhibited A549 cell growth to a much lesser extent (Fig. 1, column 1).

Although these results indicate that the antiproliferative effect of 20AS1 (4G) involves a sequence-specific antisense mechanism, as previously reported,[11–15] cell growth inhibition did not necessarily depend on a se-

Table I. Sequences of Synthetic Oligonucleotides

No.	Abbreviation	Sequence
1.	20AS1 (4G)[a]	AAG CTA ACG TTG AGG GGC AT
2.	20AS2	G TTG GTG AAG CTA ACG TTG A
3.	20S[b]	*ATG* CCC CTC AAC GTT AGC TT
4.	20SCR1 (4G)[c]	AGC ATG GTA CAA TGG GGA TC
5.	20SCR2 (2G)	AGC ATG GTG ACG TAA GGA TC
6.	20SCR3 (4C)	AGC ATG GTA CAA TCC CCA TC
7.	15AS3 (4G)	A ACG TTG AGG GGC AT
8.	15SCR (4G)	G GTA CAA TGG GGA TC
9.	10SCR (4G)	AA TGG GGA TC
10.	4G	GG GG

a) AS, antisense.
b) S, sense: *ATG* indicates the translation initiation codon.
c) SCR, oligonucleotide which has a scrambled sequence.

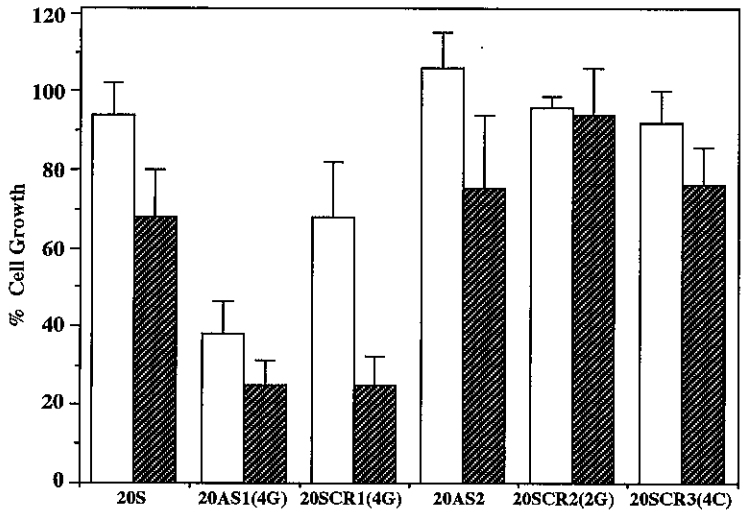


Fig. 1. Contiguous four-guanosine (4G) sequence in phosphorothioate oligonucleotide is responsible for A549 cell growth inhibition. A549 cells were cultured with various oligonucleotides at doses of 1 μM (□) and 10 μM (▨). Viable cells were counted after 5-day incubation with oligonucleotide. Untreated A549 cells were used as the control (100%). Data represent mean±SD in triplicate experiments.

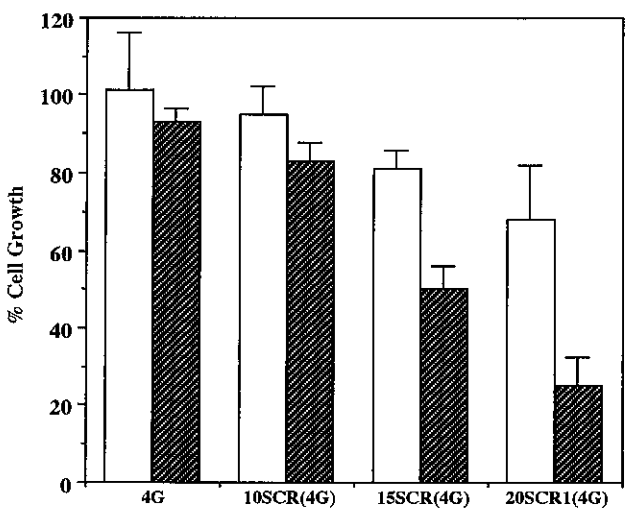
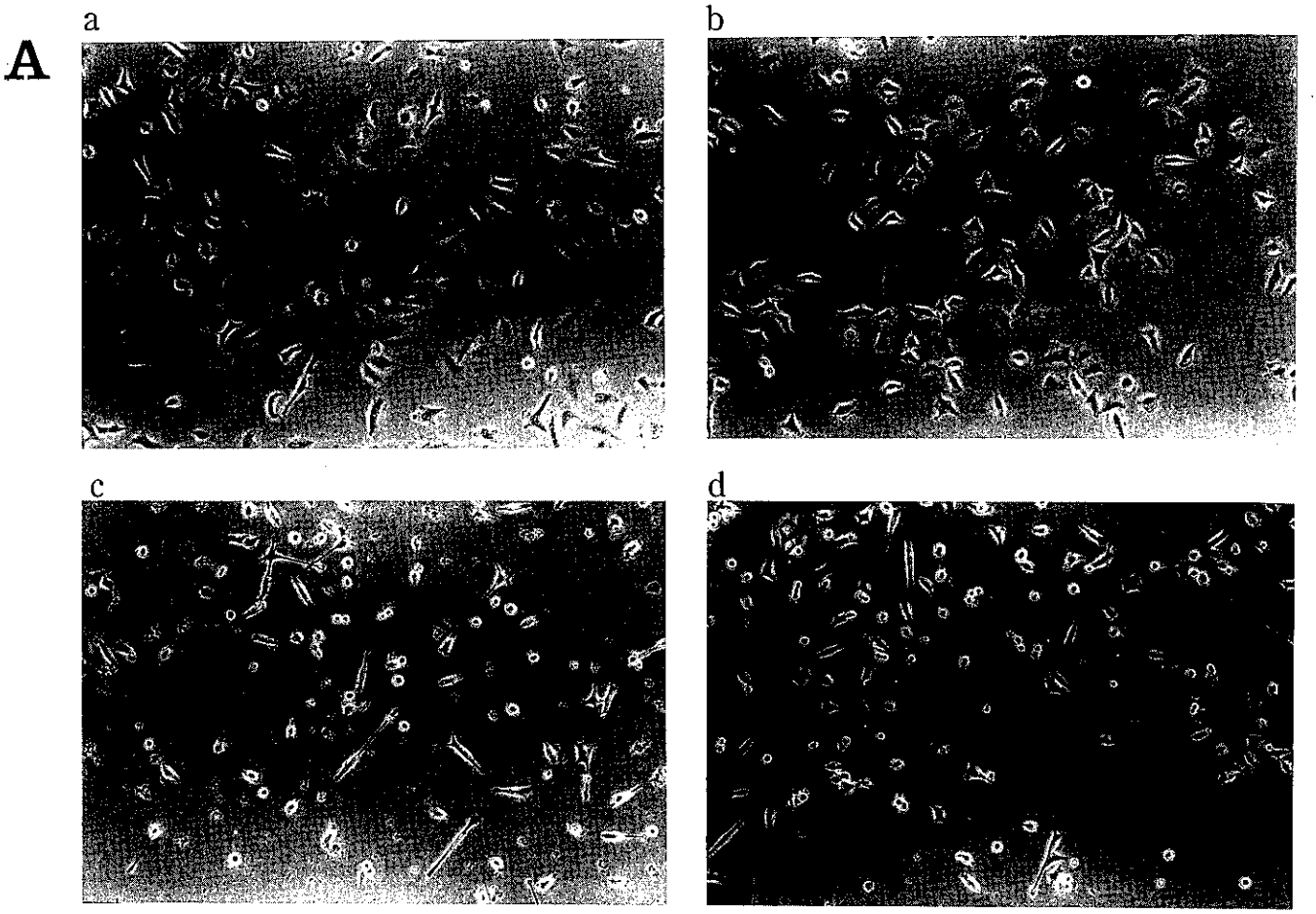


Fig. 2. Oligomer-length-dependent A549 cell growth inhibition by phosphorothioate oligonucleotides containing the contiguous four-guanosine (4G) sequence. A549 cells were cultured with phosphorothioate oligonucleotides of various lengths containing the contiguous four-guanosine (4G) sequence at doses of 1 μM (□) and 10 μM (▨). Viable cells were counted after 5-day incubation with oligonucleotides. Untreated A549 cells were used as the control (100%).

quence-specific antisense mechanism when a group of 20-mer oligonucleotides with various sequences (Table I) was used. A scrambled oligonucleotide which includes the contiguous four-guanosine sequence (20SCR1 (4G)) also inhibited cell growth as much as 20AS1 (4G) at 10 μM (Fig. 1, column 3). Furthermore, 20AS2, which has a reversed complementary sequence of the immediate downstream region of the c-myc translation initiation codon and lacks the contiguous four-guanosine (4G) sequence (Table I), was far less inhibitory than 20AS1 (4G) (Fig. 1, column 4). Neither 20SCR2 (2G) (a scrambled oligonucleotide containing a two-guanosine sequence) nor 20SCR3 (4C) (a scrambled oligonucleotide containing the contiguous four-cytidine sequence) inhibited A549 cell growth (Fig. 1, columns 5, 6). These results demonstrated that growth inhibition of A549 cells by c-myc antisense phosphorothioate oligonucleotides was not specific to the antisense sequence, but rather depended upon the contiguous four-guanosine (4G) sequence.

Growth inhibition of A549 cells by oligonucleotides containing the contiguous four-guanosine (4G) sequence is dependent on oligomer length Oligonucleotides of various lengths containing the contiguous four-guanosine (4G) sequence were synthesized, and cell growth inhibition by these oligonucleotides was studied (Fig. 2). The 15-mer (15SCR (4G)) and 20-mer (20SCR1 (4G)) oligonucleotides inhibited cell growth by 47% and 76% at 10 μM, whereas the 4-mer (4G) and 10-mer (10SCR (4G)) inhibited cell growth only by 7% and 16% ($P<0.01$), respectively. Interestingly, cell growth inhibition of 15AS3 (4G) (a 15-mer oligonucleotide with 4G and an antisense sequence) was as effective as that of 20AS1



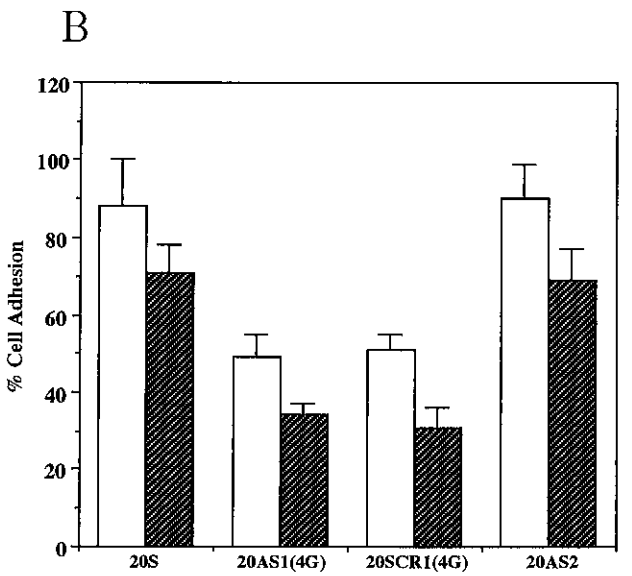


Fig. 3. Inhibition of cell adhesion by phosphorothioate oligonucleotides containing the contiguous four-guanosine (4 G) sequence. A, Morphologic alteration of A549 cells after 24-h treatment with phosphorothioate oligonucleotides. (a) control (no treatment), (b) 20S, (c) 20AS1 (4G), (d) 20-SCR1 (4G). B, Effect of phosphorothioate oligonucleotides containing the contiguous four-guanosine (4G) sequence on cell attachment to a plastic substratum. Adhesion inhibition was determined after 15-h treatment with phosphorothioate oligonucleotides as described in "Materials and Methods." Data represent mean±SD in triplicate experiments.

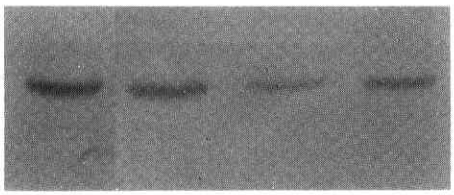


Fig. 4. Western blot analysis of c-myc protein expression after treatment with oligonucleotide. A549 cells treated with oligonucleotides at 10 μM for 24 h were lysed, and electrophoresed, and then transferred to a nylon membrane. The blot was developed as described in "Materials and Methods." Line 1, no treatment (control); line 2, 20S; line 3, 20AS1 (4G); line 4, 20SCR1 (4G).

Table II. Effect of Oligonucleotide Sequence on Cell Growth, Cell Adhesion, and c-myc Protein Expression of A549 Cells[a)]

Oligonucleotide	Growth (%)[b)]	Adhesion (%)[b)]	c-myc protein (%)[c)]
20S	68±12	72±8	92
20AS1 (4G)	23±7	33±3	38
20SCR1 (4G)	24±10	28±6	68

a) A549 cells were treated with oligonucleotides at 10 μM.
b) Data represent mean±SD of triplicate experiments.
c) Data represent mean of duplicate experiments.
Values of cell growth, adhesion and c-myc protein expression in the absence of oligonucleotide were taken as 100%.

Table III. Discrepancy of Relative c-myc Expression before Oligonucleotide Treatment and IC_{50} of c-myc 20AS1 (4G) on Human Lung Cancer Cell Lines

Cell line	Original histology	c-*myc* mRNA expression[a)]	IC_{50} (μM)
Lu65	sm[b)]	4.9	>20
SBC-3	sm	5.4	<0.5
A549	ad[c)]	1.0	0.5±0.2
RERFLCMS	ad	1.8	0.6±0.1
HS-24	sq[d)]	2.2	<0.5
Sq-19	sq	1.1	<0.5

a) Data represent mean of duplicate experiments.
b) Small cell carcinoma.
c) Adenocarcinoma.
d) Squamous cell carcinoma.

(4G) (71% and 77%, respectively). These results demonstrated that cell growth inhibition by phosphorothioate oligonucleotides containing a contiguous four-guanosine (4G) sequence was dependent on the length of the oligonucleotide. Thus, the contiguous four-guanosine (4G) sequence was essential but insufficient to inhibit cell growth, and a phosphorothioate oligonucleotide containing the contiguous four-guanosine (4G) sequence should be a 15-mer or larger to show significant cell growth inhibition.

Inhibition of A549 cell adhesion by phosphorothioate oligonucleotides containing the contiguous four-guanosine (4G) sequence Treatment of 20AS1 (4G) and 20SCR1 (4G) oligonucleotides changed the cell morphology to round, and these cells were readily detachable from the flask substratum (Fig. 3A, c,d). The 20S oligonucleotide did not alter the cellular morphology or the capability of attachment to the flask (Fig. 3A, b). Inhibition of cell adhesion was quantitated after 15-h treatment with various phosphorothioate oligonucleotides at 10 μM. Treatment with either 20AS1 (4G) or 20SCR1 (4G) oligonucleotide caused about 70% inhibition of cell adhesion (Fig. 3B), while 20S and 20AS2 oligonucleotides showed minimal inhibitory effects on cell adhesion (Fig. 3B). Inhibition of cell adhesion was dose-dependent. These results suggest that inhibition of cell adhesion was related to the contiguous four-guanosine (4G) sequence, but not to the antisense sequence.

Effect of oligonucleotide sequence on A549 cell growth, cell adhesion, and c-myc protein expression Suppression of c-myc protein expression was studied by western blot analysis after 24-h treatment with oligonucleotides at 10 μM (Fig. 4). c-myc protein was strongly suppressed only by 20AS1 (4G) treatment (Fig. 4, line 3). Table II summarizes the data on cell growth, cell adhesion, and c-myc protein expression. Cell growth inhibition was apparently correlated with adhesion inhibition when 20AS1 (4G) and 20SCR1 (4G) were used. In contrast, the expression of c-myc protein was strongly suppressed (by 62%) by 20AS1 (4G), while 20S and 20SCR1 (4G) treatment showed only 8% and 32% suppression, respectively.

Discrepancy of relative c-*myc* mRNA expression and IC_{50} of 20AS1 (4G) on human lung cancer cell lines The oligonucleotide concentration which showed 50% cell growth inhibition (IC_{50}) was determined after 5-day treatment with 20AS1 (4G). The relative c-*myc* mRNA expression before oligonucleotide treatment of each cell line was determined, based on the expression of c-*myc* mRNA in A549 cells taken as 1.0. Although c-*myc* gene amplification or c-myc overexpression compared to the nontransformed cell line has not been evaluated for all the cell lines used, Lu65 cells and SBC-3 cells show about 10-fold overexpression of the c-*myc* gene.[20-22] Therefore, c-myc might be overexpressed in all the cell lines used in this study. The relative c-*myc* mRNA expression before treatment and IC_{50} are summarized in Table III. There

was no correlation between c-myc expression and IC_{50}. For example, although IC_{50} of SBC-3 and that of Sq-19 were similar, SBC-3 expressed the highest level of c-*myc* mRNA, while Sq-19 expressed the lowest. Lu65 cells were highly resistant to 20AS1(4G) oligonucleotide treatment. This is probably because Lu65 cells could grow in suspension form, despite the fact that adhesion inhibition of Lu65 cells was similar to that in other cell lines. In contrast, other cell lines, including A549, were unable to grow in suspension form. 20SCR1 (4G) had a similar IC_{50} to that of 20AS1 (4G) (data not shown).

DISCUSSION

In this study, we demonstrated that the anti-proliferative effect of c-myc antisense phosphorothioate oligonucleotides on human lung cancer cell lines was not specific to the antisense sequence, and that this effect was rather caused by adhesion inhibition.

Modified phosphorothioate oligonucleotides have been used extensively to inhibit gene expression.[2] The biological effect could result from both sequence-specific and sequence-non-specific mechanisms.[7, 16, 23, 24] The phosphorothioate oligonucleotides are highly charged polyamines which can bind to various proteins such as basic fibroblast growth factor (bFGF) and acidic FGF, and this may contribute to the non-antisense effect.[25]

Inhibition of c-myc expression by phosphorothioate oligonucleotides targeted to the translation initiation codon of c-*myc* mRNA was found to inhibit cell proliferation of a variety of cells, including human smooth muscle cells,[11] a human leukemic cell line HL-60,[12] and a human breast cancer cell line MCF-7.[13] These antisense oligonucleotides targeted to the translation initiation codon include the contiguous four-guanosine (4G) sequence. In previous reports on the antiproliferative effect of c-myc antisense oligonucleotides, however, the control oligonucleotide did not contain a contiguous four-guanosine (4G) sequence.[11–13] Recently, Burgess *et al.*[16] reported that the antiproliferative effect of c-myc antisense oligonucleotides in smooth muscle cells was caused by a non-antisense mechanism, and proposed that the contiguous four-guanosine (4G) sequence was responsible for the antiproliferative effect. There is only one report showing that a chicken c-myc antisense oligonucleotide not containing the contiguous four-guanosine (4G) sequence inhibited cell proliferation of chicken chondrocytes *in vitro*.[26] However, the antisense sequence is targeted to the translation initiation site for chicken c-*myc* gene (*ATG* CCG CTC AGC GCC), and it is not clear whether internal GGCG and GCGG in the antisense sequence would exert any effect on cell growth.

Here, we have confirmed that growth inhibition of human lung cancer cells by c-myc antisense oligonucleotides was antisense sequence-non-specific, and was due to the contiguous four-guanosine (4G) sequence. Although 20SCR1 (4G) had an equal cell growth inhibition effect to 20AS1 (4G) at 10 μM, suppression of c-myc protein expression by 20AS1 (4G) was stronger than that by 20SCR1 (4G) (Fig. 4, Table II). This may suggest that inhibition of c-myc expression is not likely to be related to antiproliferative effect. In contrast, at a low dose (1 μM), 20AS1 (4G) showed stronger cell growth inhibition than 20SCR1 (4G) (Fig. 1). This stronger cell growth inhibition by 20AS1 (4G) at low dose was also observed with the 15-mer antisense oligonucleotide (15AS3 (4G) vs. 15SCR (4G)) (data not shown). Furthermore, low dose treatment of 20AS1 (4G) (1 μM) inhibited c-myc expression by 58%. In view of the stronger antiproliferative effects at low dose and stronger inhibition of c-myc expression by 20AS1 (4G) than 20SCR1 (4G), a true antisense effect of c-myc antisense oligonucleotides could be exerted at lower doses.

In addition to inhibition of cell growth and expression of c-myc protein, inhibition of cell adhesion and morphological changes were also caused by oligonucleotides with the contiguous four-guanosine (4G) sequence. The human lung cancer cells used in this study all grow in an adherent mode, except Lu65. Lu65 cells could grow both in adherent form and in suspension.[27] Despite the fact that adhesion of Lu65 cells was inhibited as much as that of other cell lines, Lu65 cells were able to grow in suspension form, resulting in apparent resistance to oligonucleotides containing the GGGG sequence (Table III). Inhibition of cell adhesion by c-myc antisense phosphorothioate oligonucleotides was reported by Watson *et al.*[28] They delivered oligonucleotides to MCF-7 cells by electroporation, and demonstrated that cell adhesion was strongly inhibited. An anti-adhesive effect was also reported by Perez *et al.* using an antisense phosphorothioate oligonucleotide targeted to the p65 (RelA) subunit of NF-κb, a nuclear transcription factor.[29] Interestingly, this antisense oligonucleotide did contain a contiguous four-guanosine (4G) sequence, whereas the control oligonucleotide did not. In the subsequent study, they demonstrated that antisense specificity is determined not only by the content of the sequence, but also by relation to the surrounding sequences by using serial mismatch oligonucleotides.[30] Despite their extensive study, the specificity of antisense oligonucleotides for *p65* gene remains unclear. Although it is also unclear whether inhibition of cell adhesion by oligonucleotides containing the contiguous four-guanosine (4G) sequence is a primary event or a secondary event due to cell death in this study, it has been demonstrated that inhibition of cell adhesion by blocking of integrin-mediated adhesion by peptides results in apoptosis.[31] Thus, the anti-adhesive effect is likely to be a primary event.

Various biological effects have been ascribed to oligonucleotides containing the 4G sequence, including antiproliferative effects,[16] antiviral effects,[8] and inhibition of specific enzymatic activities.[32] As in this study, antiproliferative effects were reported in many cells, but not all cells.[33] Wyatt *et al.* reported that a guanosine quartet structure was responsible for antiviral effects.[34] Phospholipase A_2 activity was inhibited by oligonucleotides containing at least two sets of three or more consecutive guanosine residues.[32] Interestingly, Smith *et al.* demonstrated that $G_3T_4G_3$ oligonucleotides form an asymmetric, diagonally looped dimeric quadruplex structure.[35] Whether these unique structural motifs are responsible for some or all of the biological effects remains to be elucidated. Our present studies indicated that usefulness of c-myc antisense phosphorothioate oligonucleotides must be evaluated with caution.

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