Design of Potent Phosphorothioate Antisense Oligonucleotides Directed to Human Interleukin 10 Gene Product and Their Evaluation of Antisense Activity in U937 Cells

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Purpose. The two objectives of this study were to design potent phosphorothioate antisense oligonucleotides (AS-S-oligos) directed against the human interleukin-10 (IL-10) gene product and to reveal the DNA sequence which best activates antisense effects.

Methods. The design of potent AS-S-oligo was performed by using melting temperature (Tm) value of a DNA/RNA hybrid calculated by the nearest neighbor method and a secondary structure of human IL-10 mRNA suggested by RNA folding algorithms. U937 cells were used to estimate the antisense effect of the AS-S-oligos.

Results. Of the eight candidates selected as potent AS-S-oligos on the basis of having higher Tm values and favorable secondary structures of the IL-10 mRNA, AS-S-oligos directed against the translated (AS367-S-oligo) and 3′-untranslated (AS637-S-oligo) region of IL-10 mRNA showed the strongest inhibitory effects on IL-10 production and this inhibition was dose- and time-dependent. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that the antisense effects of AS-S-oligos originated from a specific reduction of target IL-10 mRNA by hybridization with AS367- and AS637-S-oligos. In addition, these AS-S-oligos did not affect human tumor necrosis factor-α (TNF-α) production in the cells stimulated by lipopolysaccharide (LPS). Strong positive correlations between the inhibitory effect of AS-S-oligos on the IL-10 production and not only Tm values calculated by nearest neighbor method but also Tm values determined by absorbance versus temperature profiles were demonstrated except for AS25-S-oligo and AS1249-S-oligo.

Conclusions. These findings suggest AS367- and AS637-S-oligos powerfully inhibit IL-10 production in U937 cells via an antisense mechanism. In addition, it is suggested efficiency of AS-S-oligo directed against the sequence of the target gene product can be explained by these Tm values and the proposed secondary structures of the target gene product.

KEY WORDS: antisense oligonucleotide; interleukin-10; antisense effect; melting temperature; secondary structure.

INTRODUCTION

Interleukin-10 (IL-10) was originally described as a product of $CD4^+$ Th2 lymphocytes (Th2 cells), which was capable of suppressing cytokine secretion by $CD4^+$ Th1 lymphocytes (Th1 cells) (1). Subsequent studies have shown IL-10 is synthesized by multiple cell types including monocytes, mast cells, B cells, as well as a subset of activated human Th1 cell lines (2) and Tr1 cells (3). IL-10 is a prominent immunoregulatory and anti-inflammatory cytokine with multiple biological properties, although IL-10 was initially characterized as a counter-regulatory cytokine for Th1 development (4).

From the clinical point of view, it is well known IL-10 is associated with the chronization of atopic dermatitis (5), the abation of antitumor responses (6) and delayed immunosuppression in multiple organ dysfunction syndrome such as immunodepression after brain injury (7), suggesting the regulation of IL-10 production may be a potential target for immunotherapeutic intervention in atopic dermatitis, tumor, and immunodepression after brain injury.

Antisense oligonucleotides (AS-oligos) have provided the potential for a sequence-specific inhibitory effect of a gene expression in both animal models and in selective human clinical trials. Noteworthy, Vitravene™ (formivirsen sodium), the first drug in a class of novel therapeutics based on antisense technology for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS, was approved for marketing by the United States Food and Drug Administration in 1998. Moreover, the basic medicinal chemistry for the next generation of antisense drugs has been created.

We previously reported phosphorothioated antisense oligonucleotides (AS-S-oligos) designed to hybridize specifically to murine IL-10 mRNA have been shown to inhibit the expression in RAW264.7 cells, a murine macrophage-like cell line (8). However, the clinical use of AS-S-oligo for murine IL-10 is difficult due to a difference in sequence between murine IL-10 and human IL-10 mRNA (9). Thus, there is a need to design antisense oligonucleotides (AS-oligos) for human IL-10 mRNA. So far, there have been only a few reports of the use of AS-oligos against human IL-10 mRNA; 1) AS-S-oligo (position 313–339) abated the IL-10 mRNA level and inhibited the growth of malignant $CD5^+$ B cells (10), 2) AS-oligo (position 31–50) abated the IL-10 mRNA and IL-10 levels in human immunodeficiency virus-associated B-lymphoma cells (11) and 3) AS-S-oligo (position 25–39) not only abated the IL-10 mRNA and IL-10 levels but also induced HLA-D locus genes in peripheral blood mononuclear cells (PBMC) derived from MHC class II-deficient patients (12). Thus, the sequences of AS-oligos used in these three studies were all different, and it is not clear exactly how these AS-oligos were selected.

In the present study, we therefore designed eight 18-mer AS-S-oligos corresponding to sequences in the neighborhood of the AUG initiation codon, open reading frame and 3′-untranslated region of the human IL-10 mRNA by using Tm value of AS-S-oligo/target hybrid and a predicted secondary structure of human IL-10 mRNA. Then, we examined the effects of these AS-S-oligos on IL-10 production in U937 cells, a human histiocytic lymphoma cell line, and in this paper we also discuss the relationship between the Tm values and the inhibitory effects of AS-S-oligos on the human IL-10 production.

MATERIALS AND METHODS

Chemicals and Reagents

Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) from Escherichia coli (serotype 0111:B4) were

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purchased from Sigma (St. Louis, MO). RPMI-1640 culture medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). ISOGEN reagent and DNase I were obtained from Nippon Gene (Toyama, Japan). Reverse transcriptase (SuperScript II) and Taq polymerase (AmpliTaq Gold) were purchased from GibcoBRL (Gaithersburg, MD) and Applied Biosystems (Tokyo, Japan), respectively. Low melting temperature agarose (NuSieve GTG Agarose) was obtained from FMC BioProducts (Rockland, ME). All other chemicals and solvents were of analytical reagent grade.

Oligonucleotides

All S-oligos and oligonucleotide primers for RT-PCR as described below purified by high performance liquid chromatography (HPLC) were purchased from Pharmacia Biotech (Tokyo, Japan).

Tm Values and Secondary Structure of RNA

Tm values of DNA/RNA hybrid were calculated by using the following equation.

$$Tm^{-1} = [R \ln(Ct/n) + \Delta S^\circ]/\Delta H^\circ$$

where Tm is the melting temperature of a double helix, ΔH° and ΔS° are enthalpy and entropy changes for duplex formation estimated by Sugimoto *et al.* (13), respectively, R is the gas constant, Ct is the total strand concentration, and n reflects the symmetry factor. In addition, Tm values were also determined from absorbance and temperature curves (14). AS-S-oligo and its complementary oligodeoxyribonucleotide target were combined at 1 μM each strand in phosphate buffer (1 M NaCl, 10 mM Na_2HPO_4 and 1mM Na_2EDTA, pH 7). Secondary structure of IL-10 mRNA was predicted by MulFold software (version 2. 0d85), a MacOS port of Michael Zucker's MFold which uses an free energy minimization algorithm to predict stem and loop regions of RNA structures.

Cell Culture

U937 cells were supplied by the Japanese Cancer Research Resources Bank and maintained in suspension in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% of CO_2 in air. For differentiation to macrophages, U937 cells were plated at 1×10^6 cells/ml in the medium containing 10 ng/ml PMA and allowed to adhere for 12 hr, after which they were fed with PMA-free medium and cultured for 48 hr prior to use. The U937 cells (1×10^5 or 2×10^6 cells/well) were preincubated with various S-oligos at the indicated concentrations for 4 hr in fetal calf serum (FCS)-free RPMI-1640 culture medium. Then, the U937 cells were further incubated for the indicated times with or without 100 μg/ml of LPS in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS to elicit the production of human IL-10, IL-10 mRNA, β-actin mRNA and TNF-α. The viability of U937 cells was never less than 95%.

Determination of IL-10 and Tumor Necrosis Factor-α (TNF-α)

The level of IL-10 in cell-free supernatants was routinely assayed by a sandwich ELISA using pairs of purified capture and biotinylated detection mAbs recognizing human IL-10 (PharMingen, San Diego, CA) according to the manufacturer's protocols. The level of human TNF-α was also assayed by a sandwich ELISA using commercial kits (R&D systems, MN).

Detection of IL-10 mRNA by RT-PCR Analysis

Total RNA was isolated from U937 cells by the guanidinium-isothiocyanate procedure. Briefly, U937 cells (2×10^6 cells/well) were scraped and lyzed with 1 ml of ISOGEN reagent. Chloroform (0.2 ml) was added to the cell lysate, the mixed suspension was centrifuged at 12,000 g for 15 min at 4°C, and total RNA was recovered in the aqueous phase. Total RNA was precipitated by addition of 0.5 ml of isopropanol and washed once with 80% ethanol, dried, and dissolved in 10 μl of DEPC-treated water. Then, the samples were treated with DNase I (0.1 units) for 30 min at 37°C. After extraction of total RNA, cDNA was synthesized by reverse transcription using human IL-10 gene reverse primer (5′-CTGCTCCACGGCCTTGCTCTTGTT-3′) or human β-actin gene reverse primer (5′-GAAGCATTTGCGGTGGACGAT-3′) and reverse transcriptase (SuperScript II). Approximately, 2.5 μM human IL-10 or β-actin gene reverse primer was annealed to 1 μg of total RNA and extended with reverse transcriptase (200 units) in a buffer containing 4 μl of first strand buffer, 0.5 mM deoxynucleotide triphosphates, and RNase inhibitor (20 units) for 50 min at 42°C. PCR amplification was carried out in a Perkin Elmer Sciex DNA Thermal Cycler (Emeryville, CA). PCR was conducted in a total volume of 50 μl with 10 μl of cDNA, 0.5 mM of primers, 0.2 mM of deoxynucleotide triphosphates, and 1.25 units of Taq DNA polymerase (AmpliTaq Gold). The PCR amplimer for the human IL-10 gene was the forward primer (5′-AGGCATGCACAGCTCAGCACTGCT-3′), and for the human β-actin gene was the forward primer (5′-TCCTGTGGCATCCACGAAACT-3′). PCR was performed for 45 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 120 s, and extension at 72°C for 180 s. The amplified products were analyzed on low melting temperature agarose gels containing 0.1 μg/ml ethidium bromide.

Statistics

Data are given as means ± SEM. Statistical significance of mean comparisons was determined by analysis of Duncan's test to control for multiple comparison. P values for significance were set at 0.05. Statistical analysis was calculated on a PC-9801-F personal computer.

RESULTS

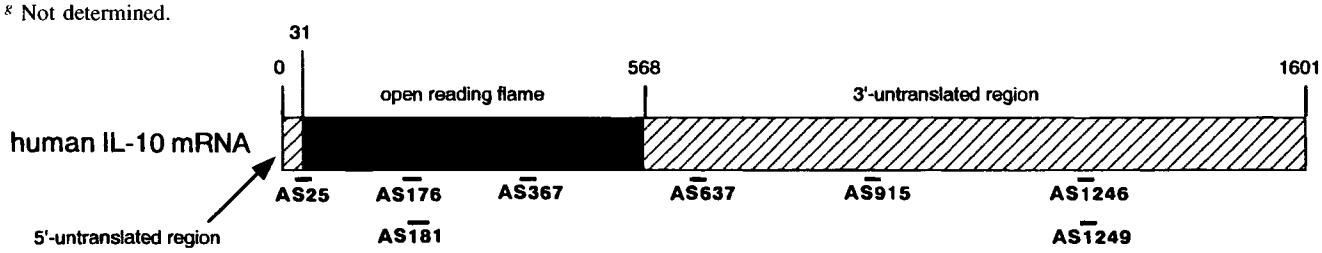
Design of AS-S-oligos

Table 1 depicts the eight candidate for potent AS-S-oligos directed against the sequences of human IL-10 mRNA and the four types of control S-oligos used in the present study. These AS-S-oligos were designed with regard to a number of criteria including 1) a relative higher Tm value of AS-oligo/target mRNA, 2) a favorable secondary structure of target mRNA directed against the sequence of AS-oligo, 3) the lack of self-complementarity within chosen oligo sequences, 4) the lack of cross-hybridization to other sites within the IL-10 mRNA and

Table 1. Sequences, cDNA Targets, and Melting Temperatures of Oligonucleotides

ODNs[a]	Sequence (S-oligo, 18 mer)	Position	Tm value (°C)[b]	Tm value (°C)[c]
AS25	5′ TGA GCT GTG CAT GCC TTC 3′	25~42	76.7	58.8
AS176	5′ AGA AAG TCT TCA CTC TGC 3′	176~193	65.9	53.0
AS181	5′ TTG AAA GAA AGT CTT CAC 3′	181~198	56.4	43.2
AS367	5′ GGT CTT CAG GTT CTC CCC 3′	367~384	82.9	54.1
AS637	5′ CTG GGT CAG CTA TCC CAG 3′	637~654	78.9	58.0
AS915	5′ GCT TGG AAT GGA AGC TTC 3′	915~932	66.2	53.6
AS1246	5′ GGC TGG TTA GGA ACT CCT 3′	1246~1263	74.7	53.2
AS1249	5′ CCA GGC TGG TTA GGA ACT 3′	1249~1266	72.8	50.5
SC367[d]	5′ CTC CTG CTC AGT TGG TCC 3′	—[f]	—[f]	—[g]
SC637[e]	5′ CCA GTG TCT GCA GAG CTC 3′	—[f]	—[f]	—[g]
MC1	5′ AGG TCC TGG AGT CCA GCA 3′	—[f]	—[f]	—[g]
MC2	5′ ATG CGC GAG TAC GCG TAC 3′	—[f]	—[f]	—[g]

[a] All oligonucleotides were phosphorothioate type.
[b] Tm values of the hybrids with DNA/RNA were calculated from thermodynamic parameters based on the nearest-neighbor model (Sugimoto *et al., Biochemistry* 34:11211 (1995)).
[c] Tm values of the hybrid with DNA/DNA determined by UV method.
[d] Twelve-mismatch oligo of AS367.
[e] Fourteen-mismatch oligo of AS637.
[f] Not calculated.
[g] Not determined.



to other nontarget mRNA, and 5) the lack of sequences such as G-quartet motif (15,16) and CpG motif (17) in AS-S-oligos. Table 1 depicts two kinds of Tm values: the calculated Tm value of AS-S-oligo/oligoribonucleotide (AS-S-oligo/RNA) hybrid and the observed Tm value of AS-S-oligo/phosphodiester oligodeoxynucleotide (AS-S-oligo/DNA) hybrid. Here, the former Tm values of AS-S-oligos with human IL-10 mRNA were calculated from thermodynamic parameters based on the nearest neighbor model reported by Sugimoto *et al.* (13), although this model was established for DNA/RNA hybrid, because AS-S-oligo/RNA hybrid has a sequence-dependent Tm value that is relatively the same as that calculated using the model (18). While the latter Tm values of AS-S-oligo/DNA hybrid were determined by absorbance versus temperature profiles (14). The reason why AS-S-oligo/DNA hybrid, and not AS-S-oligo/RNA hybrid, was used, is that the use of AS-S-oligo/DNA hybrid is convenient when the sequence of AS-oligo is screened. The calculated and observed Tm values ranged from ca. 56 to 83°C and 43 to 59°C, respectively (Table 1). Thus, the calculated Tm values are higher than the observed values. The differences in these Tm values may be explained as follows, i.e., Tm value of DNA/RNA hybrid is higher than DNA/DNA hybrid and the diastereomer of AS-S-oligo is, for steric reasons, responsible for the lower Tm of the AS-S-oligo/RNA hybrid relative to the DNA/RNA hybrid.

On the other hand, the proposed secondary structure of human IL-10 mRNA (28-mer fragment) corresponding to the AS-oligos bonding sites by computer RNA folding algorithm (MulFold) had a larger hairpin loop structure (Fig. 1), which has a higher affinity with AS-oligos. In addition, the lack of self-complementarity within chosen oligo sequences was confirmed by computer oligo-analysis algorithm (Primer Calculator) and the lack of cross-hybridization to other sites within the IL-10 mRNA and to other nontarget mRNA were confirmed by analysis of potential hybridization against a gene database, GenBank, using an algorithm for FASTA homology analysis. While control S-oligo SC367, a 12 mismatch of AS367-S-oligo, SC637-S-oligo, a 14 mismatch of AS637-S-oligo, MC1-and MC2-S-oligos [two types of S-oligos scrambled antisense sequence (nucleotide position 637–654) directed against mouse IL-10 mRNA] are shown in Table 1.

Effects of S-oligos on IL-10 Production

U937 cells were incubated for 4 hr with S-oligos (20 μM) in FCS-free RPMI-1640 culture medium and further incubated for the indicated time with or without LPS (100 μg/ml) in culture medium supplemented with 10% heat-inactivated FCS. IL-10 production induced by LPS (100 μg/ml) was inhibited when U937 cells were incubated with AS-176, AS-367, AS637, AS915- and AS1246-S-oligos, but only slightly inhibited when they were incubated with AS25-, AS181-, or AS1249-S-oligos (Fig. 2A). The greatest inhibitory effect was observed with AS367-S-oligo and AS637-S-oligo. However, IL-10 production was not inhibited by treatment with control SC367-, SC637-, MC1-, and MC2-S-oligos (Fig. 2B). Furthermore, AS367- and

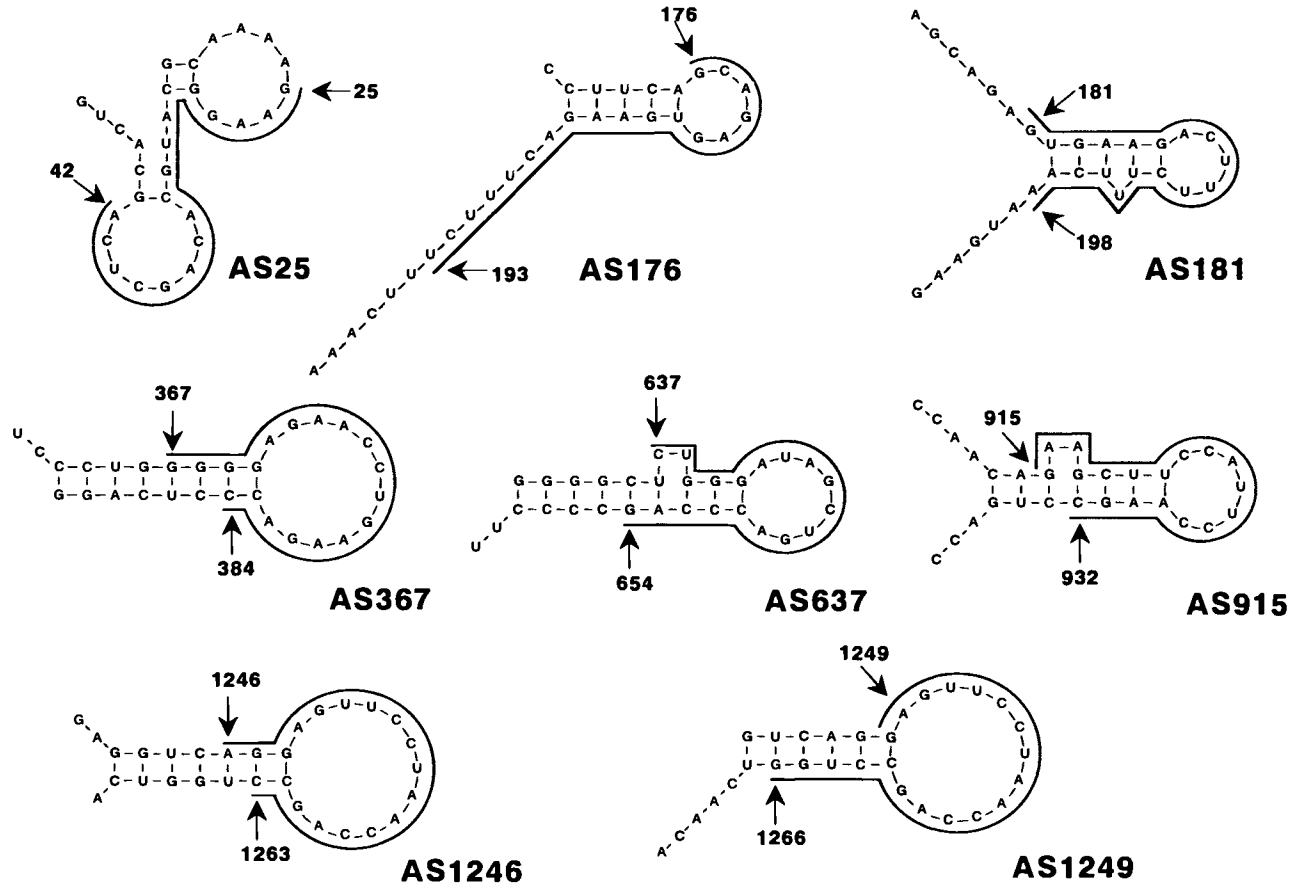


Fig. 1. Proposed secondary structure of the 28-nt fragment of human IL-10 mRNA corresponding to sequence-complementary binding sites of AS-S-oligos. The secondary structure was predicted by the MulFold computer folding program.

AS637-S-oligos showed pronounced dose- and time-dependent inhibition of IL-10 induction (Figs. 2B and 2C).

Effects of S-oligos on IL-10 mRNA Level

Figure 3 shows the IL-10 mRNA levels determined by RT-PCR of total RNA in U937 cells. We confirmed amplification of IL-10 and β-actin cDNA did not reach saturation under these experimental conditions. The 430 bp band derived from human IL-10 mRNA was observed in the extracts of U937 cells treated with two types of control S-oligos (SC367- and SC637-S-oligos) as well as in that of the positive control without S-oligos (Fig. 3A). AS367- and AS637-S-oligos caused a substantial reduction in the density of the 430 bp band but not in that of the 315 bp band derived from control β-actin mRNA (Fig. 3A). The intensity of the 430 bp band was inversely proportion to AS367-S-oligo (Fig. 3B) and AS637-S-oligo (Fig. 3C) doses, but not that of SC367-S-oligo (Fig. 3B) and SC637-S-oligo (Fig. 3C). These results indicated AS367- and AS637-S-oligos specifically decreased the IL-10 mRNA level.

Effects of S-oligos on TNF-α Production

To study the effects of AS-S-oligos on the expression of protein induced by LPS, TNF-α levels in culture supernatants were determined (Fig. 4). The extent of TNF-α induction by LPS stimulation was not substantially changed either by the four types of control S-oligos or AS367- and AS637-S-oligos (Fig. 4). These results suggest the inhibitory effects of these AS-S-oligos on IL-10 induction are target-specific.

Relationship Between Tm Value of DNA/RNA Hybrid and Inhibitory Effect of AS-S-oligos on IL-10 Production

To reveal the reason for the differences in the inhibitory effects of AS-S-oligos, we examined the relationship between the calculated and observed Tm values and the inhibitory effect (Figs. 5A and B). The results indicated with the exceptions of AS1249- and AS25-S-oligos, all other AS-S-oligos showed a positive correlation between the inhibitory effect and both the calculated Tm values (Fig. 5A) and the observed Tm values (Fig. 5B) exists, suggesting that thermally more stable duplexes have superior inhibitory effects. Thus, the most potent antisense effects of AS367- and AS637-S-oligos on IL-10 production are attributable to the higher affinity with the binding site of human IL-10 mRNA and the lack of inhibitory effects of AS181-S-oligos as well as AS1249- and AS25-S-oligos may be due to their lower affinity with the targets.

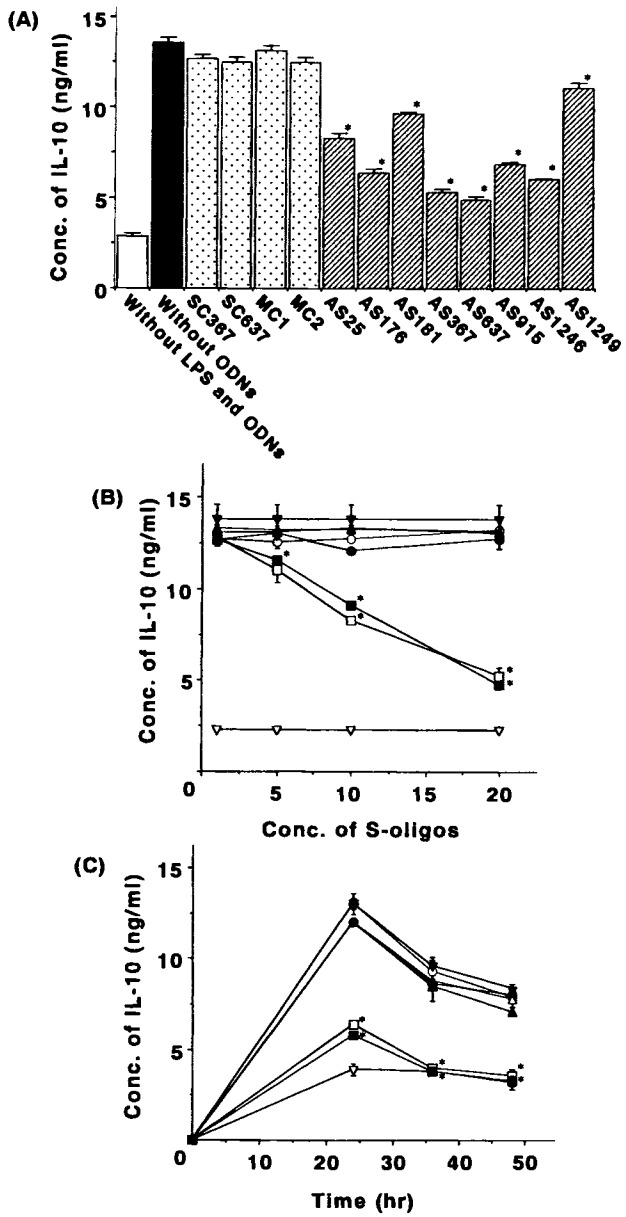


Fig. 2. Effects of AS-oligos on IL-10 production in U937 cells stimulated by LPS. (A) Comparison of inhibitory effects of AS-S-oligos on IL-10 production. U937 (1×10^5 cells) were incubated for 4 hr with AS-S-oligos (20 μM) in FCS-free RPMI-1640 culture medium and further incubated for 24 hr with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. (B) Sequence-specific inhibition of IL-10 production by AS-S-oligos. U937 (1×10^5 cells) were incubated for 4 hr with S-oligos (0, 1, 5, 10 and 20 μM) in FCS-free RPMI-1640 culture medium and further incubated for 24 hr with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactiveted FCS. ∇, without LPS and S-oligos; ▼, without S-oligos, ○, SC367-S-oligo, ●, SC637-S-oligo; △, MC1-S-oligo; ▲, MC-2-S-oligo; □, AS367-S-oligo, ■, AS637-S-oligo. (C) Time-dependent effects of AS-S-oligos on IL-10 production. U937 (1×10^5 cells) were incubated for 4 hr with AS-S-oligos (20 μM) in FCS-free RPMI-1640 culture medium and further incubated for the indicated periods with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. In these studies, IL-10 levels in the supernatants of culture medium were measured by ELISA as described in Materials and Methods. ∇, without LPS and S-oligos; ▼, without S-oligos; ○, SC367-S-oligo; ●, SC637-S-oligo; △, MC1-S-oligo; ▲, MC-2-S-oligo, □, AS367-S-oligo; ■, AS637-S-oligo. The values represent the means ± SEM of three experiments. *$p < 0.05$ vs control (without S-oligos).

DISCUSSION

Our results indicate AS367- and AS637-S-oligos show the greatest inhibitory effect on IL-10 induction among the eight 18-mer AS-S-oligos, while AS25-, AS181- and AS1249-S-oligos showed only slight inhibitory effects. The most potent antisense effects of AS367- and AS637-S-oligos on IL-10 production can be explained by the higher affinity with the binding site of human IL-10 gene product and the lack of inhibitory effects of AS181-S-oligos may have been due to the lower affinity (low Tm value) with the target.

However, why do AS1249- and AS25-S-oligos, which have a relatively high Tm values, have low inhibitory effects? Why do these AS-S-oligos deviate from the regression curve? (Fig. 5) As shown in Fig. 1, the IL-10 mRNA regions directed against AS1249-S-oligo and AS25-S-oligo sequence demonstrated a favorable secondary structure, which had a higher affinity to AS-S-oligos. Thus, it is likely the mechanism for the inferior inhibitory effects of AS1249-S-oligo and AS25-S-oligo can not be explained by the Tm values or a secondary structure of IL-10 mRNA. In addition, it is interesting to note the difference in the inhibitory effects between AS1249-S-oligo and AS1246-S-oligo, as these AS-S-oligos have similar Tm values and their target sites are very close. Thus, it is possible the lower inhibitory effect of AS1249-S-oligo might be attributable to other factors, e.g., the difference in the side of the single strand which AS-S-oligo hybridize and the influence of the local binding of RNA binding proteins with IL-10 mRNA, and/or the tertiary structures of human IL-10 mRNA. Moreover, another possible explanation for the low inhibitory effects of AS1249- and AS25-S-oligos is the extent of these AS-S-oligo's binding to proteins may have some influence on their cellular uptake and inhibitory effect on IL-10 induction. Further study on the difference in these antisense activities of AS1249-and AS25-S-oligos is required.

To date, it is reported three types of AS-S-oligos have a potent antisense activity, i.e., 1) AS(25-39)-S-oligos (target position 25–39, 15 mer) (12), 2) AS(31-50)-S-oligos (target position 31–50, 20 mer) (11) and AS(313-339)-S-oligos (target position 313–339, 27 mer) (10). However, our results indicated AS(25-39)-S-oligos changed the IL-10 production only very slightly (data not shown), although AS25-S-oligo had a moderate effect (Fig. 2). It is clear there is a difference in the antisense effects between Hauber's results (11) and our results. We have not examined the antisense activity of AS(31-50)-S-oligo and AS(313-339)-S-oligo under these experimental conditions, because the former has a similar Tm value (72.15°C, the calculated value of DNA/RNA hybrid) to AS25-S-oligo and its target has a inferior secondary structure (a small hairpin loop) and a long double strand region, whereas the latter has a G-quartet motif in the sequence as described below. These findings suggest the possibility AS367-S-oligo and AS637-S-oligo are more potent human IL-10 AS-S-oligos.

The inhibitory effects of AS367- and AS637-S-oligos on IL-10 production among the eight 18-mer AS-S-oligos in a dose- and time-dependent manner. Under the present experimental conditions, the peak of IL-10 mRNA and TNF-α mRNA levels were about 12 hr and 3 hr after LPS stimulation, respectively (data not shown). On the other hand, the superior inhibitory effects of AS367- and AS637-S-oligos on IL-10 production were observed at three time points (24, 36 and 48 hr) (Fig.

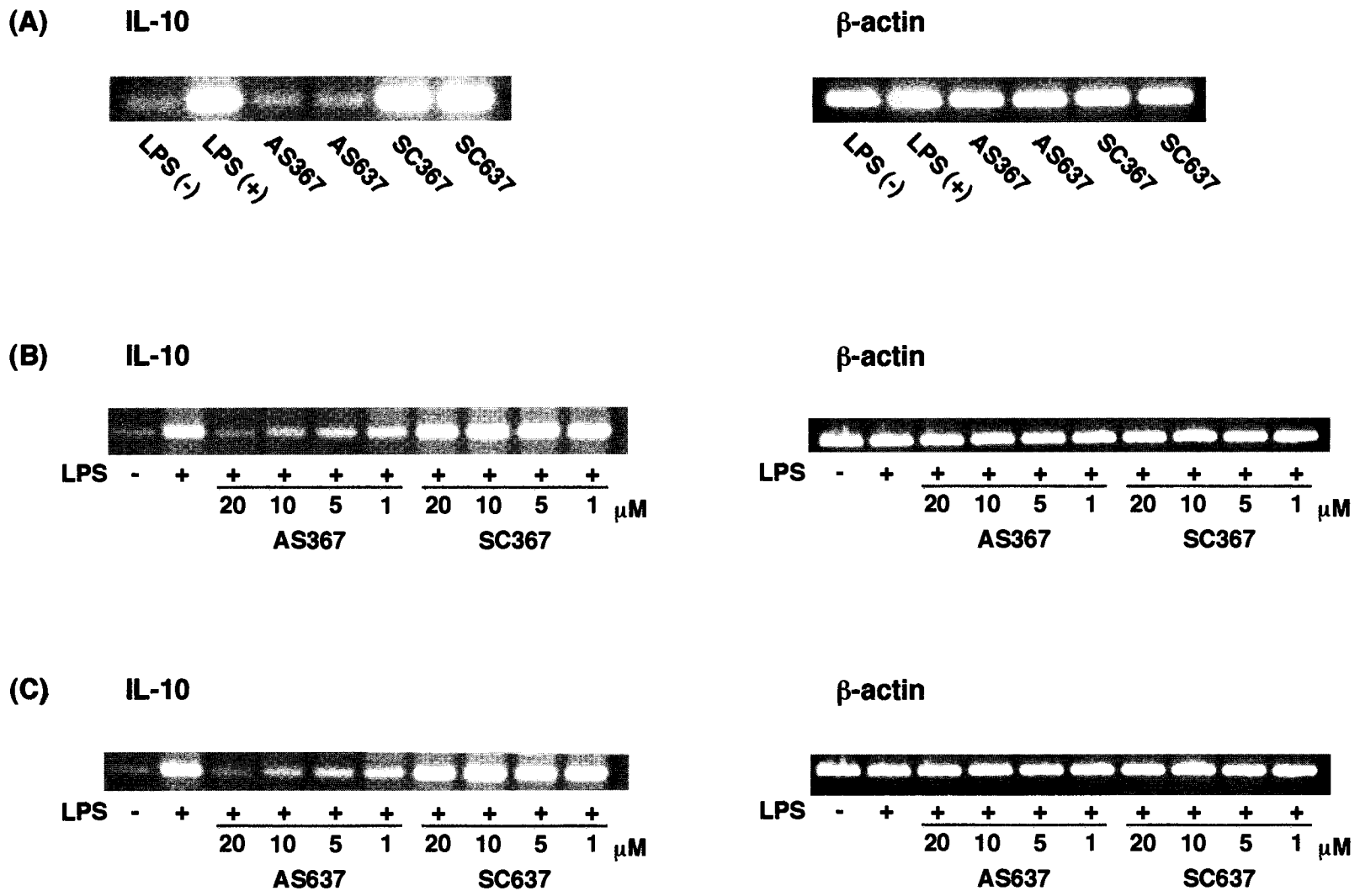


Fig. 3. Effects of AS-oligos on IL-10 mRNA expression in U937 cells stimulated by LPS. (A) Sequence-specific inhibitory effects of AS-oligos on IL-10 mRNA. U937 (2×10^6 cells) were incubated for 4 hr with S-oligos (20 μM) in FCS-free RPMI-1640 culture medium and further incubated for 24 hr with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. IL-10 mRNA and control β-actin mRNA were determined by RT-PCR as described in Materials and Methods. (B) Concentration-dependent effects of AS367-S-oligo on IL-10 production. (C) Concentration-dependent effects of AS637-S-oligo on IL-10 production. U937 cells (2×10^6 cells) were incubated for 4 hr with various concentrations of S-oligos in FCS-free RPMI-1640 culture medium and further incubated for 24 hr with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. IL-10 mRNA and control β-actin mRNA were determined by RT-PCR as described in Materials and Methods.

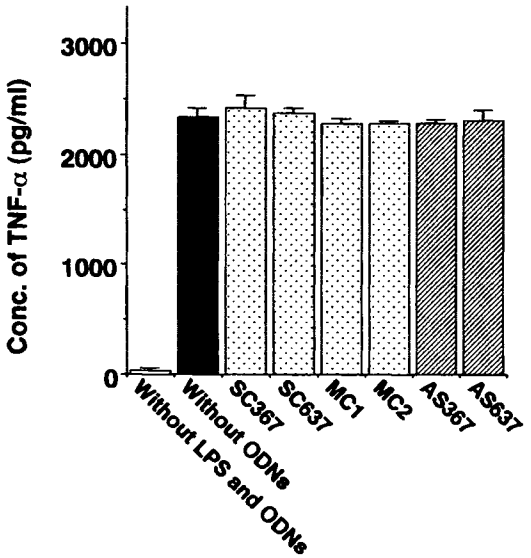


Fig. 4. The effects of S-oligos on TNF-α production. U937 cells (1×10^5 cells) were incubated for 4 hr with S-oligos (20 μM) in FCS-free RPMI-1640 culture medium and further incubated for 24 hr with or without LPS (100 μg/ml) in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. TNF-α levels in the supernatants of culture medium were measured by ELISA as described in Materials and Methods. The values represent the means ± SEM of four experiments.

2C). This sustained effect of AS367- and AS637-S-oligos can be explained by the finding which U937 cells were incubated for 4 hr with S-oligos (20 μM) in culture medium and then incubated for 24, 36, or 48 hr with LPS in culture medium: sufficient time exists to which AS-S-oligo is entered into the cells before IL-10 mRNA is inducted, and sufficient amounts of AS367- and AS637-S-oligos in culture medium and cells exist to which AS-S-oligo maintains the antisense effect during this experimental time period. Therefore, it is likely that no AS-S-oligos used here affect TNF-α production as well as IL-10, although the effects of AS-S-oligos on TNF-α production were observed only once 24 hr after the LPS stimulation in this study.

The antisense effect of AS637-S-oligo cannot be explained by translational arrest, since this AS-S-oligo was directed against the 3′-untranslated region of the IL-10 mRNA. To test this mechanism, we studied IL-10 mRNA level by using RT-PCR. AS637-S-oligo treatment decreased the mRNA level in a dose-dependent manner (Fig. 2C). Similar behavior was observed in the AS367-S-oligo treatment (Fig. 2B). On the other hand, the most generally accepted mechanism for RNA cleavage of the hybrid of AS-oligo and mRNA involves activation of RNase H, a ubiquitous enzyme that specifically degrades the RNA strand of DNA/RNA hybrids and AS-S-oligo acts as substrates for RNase H (19,20). In addition, it is well known that cytokine mRNAs, for example, IL-2 and IFN-γ posses a destabilizing sequence (UAUUUAU) in the 3′-untranslated region, eventually, human IL-10 mRNA includes the four destabilizing sequences in the 3′-untranslated region. Therefore, the results suggest the possibility that AS367- and AS637-S-oligos destabilize the IL-10 mRNA either by RNase H-dependent and-independent mechanisms.

Neither AS367-S-oligo nor AS637-S-oligo changed TNF-α production in U937 cells stimulated by LPS (Fig. 4). Thus, the results suggested that these AS-S-oligos inhibited the IL-10 production in U937 cells in a target-specific manner. This speculation may be supported by the finding that these AS-S-oligos failed to change the β-actin expression (Fig. 3) in U937 cells stimulated by LPS. Therefore, it is most likely that AS367- and AS637-S-oligos have a target-specific antisense activity.

Recently, sequence-specific and nonspecific binding of S-oligos to small molecules (21) and proteins (22) have been described. In addition, S-oligos often exhibited nonantisense effects which can be sequence-specific or nonspecific (23). It was shown that S-oligos with G-quartet motifs have antiviral (14) and antiproliferative activities (15), whereas those with the CpG motif activated B cells and induced cytokine production by lymphocytes (16). On the other hand, none of the AS-S-oligos

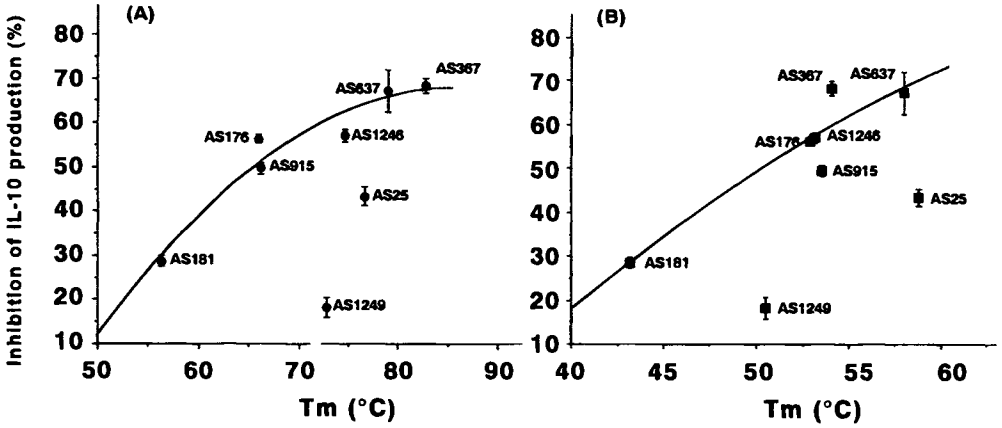


Fig. 5. Relationship between Tm values of (A) AS-oligo/RNA hybrids and (B) AS-S-oligo/DNA hybrids and the inhibitory effects of AS-S-oligos on IL-10 production. (A) Tm values of the AS-oligo/RNA hybrid calculated from thermodynamic parameters based on the nearest-neighbor model reported by Sugimoto *et al.* (13) and (B) Tm values of the AS-S-oligo/DNA hybrid as described in Materials and Methods. The inhibitory effects of AS-S-oligos on IL-10 production represents as the mean percent inhibition ± SEM of three experiments.

and control S-oligos tested here had sequences such as the G-quartet or CpG dinucleotide sequence (Table 1), so it is likely that the sequence- and target-specific antisense effects of AS367-S-oligo and AS637-S-oligo were dose-and time-dependent (Figs. 2–4).

Recently, studies in IL-10 transgenic mice and IL-10 knockout mice have revealed the pivotal role of IL-10 in the immune system: IL-10 transgenic mice show some evidence of a suppressed Th1 type response (24), are more susceptible to infection with *Mycobacterium tuberculous* and show reduced antitumor immune responses (25), whereas IL-10 knockout mice develop a severe inflammatory bowel disease (26) apparently as a consequence of a pathogenic Th1-type response (27). In addition, Wynn *et al.* (28) reported that IL-10 induction is critical for Th2 response polarization in vivo, although IL-10 is an important endogenous down-regulator of Th2 as well as Th1 cytokine synthesis. In contrast, several recent studies have suggested IL-10 itself could suppress Th2-mediated allergic inflammation (29). Thus, the role of IL-10 in immune responses is controversial. Given the above, it is interesting to evaluate the potential of AS367-and AS637-S-oligos to modulate Th2-mediated allergic inflammation such as atopic dermatitis, to augment the antitumor response and to inhibit the immunodepression induced by brain injury.

In conclusion, AS367- and AS637-S-oligos against human IL-10 mRNA have a powerful antisense activity on gene expression. In addition, the present methodology for the design of AS-S-oligo using Tm value of DNA/RNA hybrid calculated by the nearest neighbor method and the proposed secondary structure of target mRNA using a computer algorithm such as MulFold may be useful in explaining the function of cytokines. These findings suggested that AS367-and AS637-S-oligos may be useful for a specific inhibition of IL-10 production in humans.

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