2′-O-(2-Methoxy)ethyl-modified Anti-intercellular Adhesion Molecule 1 (ICAM-1) Oligonucleotides Selectively Increase the ICAM-1 mRNA Level and Inhibit Formation of the ICAM-1 Translation Initiation Complex in Human Umbilical Vein Endothelial Cells*

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Little is known about the mechanisms that account for inhibition of gene expression by antisense oligonucleotides at the level of mammalian cell biology. For this purpose, we have selected potent 2′-O-(2-methoxy)ethyl antisense oligonucleotides (IC50 = 2 and 6 nM) that target the 5′ cap region of the human intercellular adhesion molecule 1 (ICAM-1) transcript to determine their effects upon individual processes of mRNA metabolism in HUVECs. Given the functions of the 5′ cap structure throughout mRNA metabolism, antisense oligonucleotides that target the 5′ cap region of a target transcript have the potential to modulate one or more metabolic stages of the message inside the cell. In this study we found that inhibition of protein expression by these RNase H independent antisense oligonucleotides was not due to effects on splicing or transport of the ICAM-1 transcript, but due instead to selective interference with the formation of the 80 S translation initiation complex. Interestingly, these antisense oligonucleotides also caused an increase in ICAM-1 mRNA abundance in the cytoplasm. These results imply that ICAM-1 mRNA turnover is coupled in part to translation.

Antisense oligonucleotides have been shown to be effective agents for inhibition of gene expression at the mRNA level (1–3). They may be described as exogenous regulators of mRNA metabolism intended to sterically interfere with one or more metabolic processes upon hybridization, such as initiation of translation, or to promote enzyme-mediated mRNA degradation by formation or exposure of a region for nuclease activity, such as RNase H. The mode of action of an antisense oligonucleotide in cells is dependent upon its composition (sugar, backbone, and base residues) and mRNA binding site location (5′UTR, coding region, 3′UTR). Although several types of antisense oligonucleotides, which differ in composition and target site, have been found to be effective agents for sequence-specific inhibition of gene expression in mammalian cells, direct or detailed evidence of their mode(s) of action remains limited (4–9).

Intercellular adhesion molecule 1 (ICAM-1) is one of several cell adhesion molecules expressed on the cell surface of vascular endothelium that participates in a broad range of immune and inflammatory responses (10). ICAM-1 is also expressed on nonendothelial cells, such as keratinocytes, monocytes, and fibroblasts in response to inflammatory mediators. Elevated levels of ICAM-1 expression have been observed in a number of immune-related human diseases (11, 12), e.g. rheumatoid arthritis, psoriasis, and asthma. Thus, regulation of ICAM-1 gene expression is of therapeutic interest (13–15). The ICAM-1 gene has been sequenced, and the transcription initiation site has been characterized for several cell lines following induction by a variety of cytokines (16, 17), including human umbilical vein endothelial cells (HUVECs) with induction by TNF-α (18).

Previous research has demonstrated that elevated expression of ICAM-1 may be controlled in cells by phosphorothioate-modified antisense oligonucleotides (4, 5). At that time the most effective oligonucleotides were those that were compatible with RNase H and targeted the 3′UTR of the transcript. Since then advances in chemical synthesis have brought forth a number of oligonucleotide modifications at the 2′-sugar position which give significant increases in duplex stability and nuclease resistance but do not support RNase H activity (19). Antisense oligonucleotides that bind more tightly to the target mRNA are expected to be more effective at interfering with the processes of metabolism when bound at suitable locations. Bulky substrates at this position also have been shown to provide a high degree of nuclease resistance.

Biophysical and biological analysis of a set of these uniformly 2′-modified oligonucleotides (2′-O-methyl (20), 2′-O-allyl (20), 2′-O-(2-methoxy)ethyl (21), and 2′-fluoro (22)) that target the 5′ terminus of the ICAM-1 transcript led to our selection of the exceptionally active 2′-O-(2-methoxy)ethyl-modified oligonucleotide, ISIS 11158 and 11159, for an investigation of their intracellular mode of action in HUVECs (Fig. 1 and Table 1). The 5′ cap of eukaryotic mRNA has been shown to be a structural element that functions throughout mRNA metabolism (24–36). Therefore, antisense oligonucleotides which target the 5′ cap region of a designated transcript have the potential to modulate one or more metabolic stages of the message inside the cell (37). In this study the antisense mode of action was determined by evaluation of the target transcript’s metabolic processes (splicing, transport, translation, and stability) following antisense treatment and induction of gene expression.

**EXPERIMENTAL PROCEDURES**

* Cells and Cell Culture—HUVECs were purchased from Clonetics Corp. (San Diego, CA) and cultivated in the designated EBM medium.
Effects of Antisense Oligonucleotides on ICAM-1 mRNA Metabolism

A

ICAM-1 mRNA 5' UTR Sequence

5' m^7GpmpGAGCCUCUCGCUACUGAUUGCAACCUCUGCCUCGCUAUG. 3'

B

Antisense Oligonucleotide Sequence and Structures

5' TCTGATAGCAGAGGCCTC 3'

<table>
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<th>ISIS No.</th>
<th>Backbone (X)</th>
<th>2' Sugar (R)</th>
<th>Antisense activity</th>
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<td>nM</td>
<td>°C</td>
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<tr>
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<tr>
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supplemented with 10% fetal bovine serum from HyClone (Logan, UT). Cells were used for experiments from passages two to ten at 80–90% confluency.

Oligonucleotide Synthesis—Oligonucleotides were synthesized utilizing conventional solid-phase triester chemistry (38). The 2'-deoxy (Perceptive Biosystems), 2'-O-methyl (ChemGene), and 2'-O-allyl (Boehringer Mannheim) phosphoramidites were purchased from commercial sources. 2'-O-(2-Methoxy)ethyl and 2'-fluoro phosphoramidites were manufactured either in house (Dr. Bruce Ross) or under contract (R.I. Chemicals).

Oligonucleotide Treatment of HUVECs—Cells were washed three times with Opti-MEM (Life Technologies, Inc.) pre-warmed to 37 °C. Oligonucleotides were premixed with 10 µg/ml Lipofectin (Life Technologies, Inc.) in Opti-MEM, serially diluted to the desired concentrations, and applied to washed cells. Basal and untreated (no oligonucleotide) control cells were also treated with Lipofectin. Cells were incubated for 4 h at 37 °C, at which time the medium was removed and replaced with standard growth medium with or without 5 ng/ml TNF-α (R & D Systems). Incubation at 37 °C was continued until the indicated times.

Quantitation of ICAM-1 Protein Expression by Fluorescence-activated Cell Sorter—Cells were removed from plate surfaces by brief trypsinization with 0.25% trypsin in PBS. Trypsin activity was quenched with a solution of 2% bovine serum albumin and 0.2% sodium azide in PBS (+Mg/Ca). Cells were pelleted by centrifugation (1000 rpm, Beckman GPR centrifuge), resuspended in PBS, and stained with 3 µl/10^6 cells of the ICAM-1 specific antibody, CD54-PE (Becton Dickinson) and 0.1 µg of the control antibody, IgG2b-PE (Pharmingen). Antibodies were incubated with the cells for 30 min at 4 °C in the dark, under gentle agitation. Cells were washed by centrifugation procedures and then resuspended in 0.3 ml of FacsFlow buffer (Becton Dickinson) with 0.5% formaldehyde (Polysciences). Expression of cell surface ICAM-1 was then determined by flow cytometry using a Becton Dickinson FACScan. Percentage of the control ICAM-1 expression was calculated as follows: (oligonucleotide-treated ICAM-1 value) − (basal ICAM-1 value) (control-treated ICAM-1 value) − (basal ICAM-1 value).

Nuclear Runoff Transcription Analysis—Cells were treated with oligonucleotide at a concentration of 50 nM for 4 h. Cells were harvested 2 h post TNF-α induction. Preparation of nuclei and measurement of gene transcription were based upon published procedures (39). Equal counts/min of 32P-labeled RNA were hybridized to slot-blot membranes loaded with cDNA fragments for ICAM-1 and GAPDH. The 6X 174 DNA HaeIII digest was included as a control.

Total RNA Isolation and Northern Analysis—Total cellular RNA was isolated from HUVECs by lysis and precipitation with Catrimx-14 (Iowa Biotechnology Corp.), followed by extraction of the DNA from the precipitate with lithium chloride. Isolated RNA was separated on a 1.0% agarose gel containing 1.1% formaldehyde, then transferred and...
UV-crosslinked (Stratalinker 2400, Stratagene) to a Hybond N nylon membrane (Amersham). Blots were hybridized with Prime-a-Gen eDNA probes (Promega) using RapidHyb (Amersham). Probes were generated from the following human cDNA restriction or PCR fragments: a 1.88-kilobase ICAM-1 fragment (BBC 58, R & D Systems), a 1.1-kilobase G3PDH fragment (pHeGAP, American Tissue Culture Collection), and a 2-kilobase E-selectin fragment (4). A Molecular Dynamics PhosphorImager was utilized to quantitate Northern blot probe signals.

**Nuclear and Cytoplasmic RNA Fractionation**—Harvested cells were incubated in mild lysis buffer (0.5% Nonidet P-40, 10 mM Tris-Cl (pH 7.4), 140 mM KCl, 5 mM MgCl2, and 1 mM DTT) for 5 min at 4 °C (40). Nuclei were separated from the cytosol by centrifugation at 1000 × g for 5 min at 4 °C. The cytosol was transferred to a sterile tube containing 3 volumes of a denaturing solution (5.3 M guanidinium isothiocyanate, 37.5 mM sodium citrate (pH 7.0), 0.75% Sarkosyl, and 0.15 mM 2-mercaptoethanol), phenol-extracted under acidic conditions (pH 4.0), and isopropanol alcohol-precipitated. A second lysis step was performed on the collected nuclei to ensure removal of the cytosol fraction. Washed nuclei were lysed at room temperature by the addition of 1 ml of Catrimox-14 surfactant. Nuclear RNA was isolated by the LiCl extraction procedure.

**Polysome Profile Analysis**—Approximately 10^6 oligonucleotide-treated cells were pelleted, washed with PBS, and then mixed into 0.3 ml of cold lysis buffer (0.5% Nonidet P-40, 10 mM Tris-Cl (pH 7.4), 140 mM KCl, 5 mM MgCl2, 1 mM DTT, 100 μg/ml cycloheximide, and RNase inhibitor (5 Prime 3 Prime)) and incubated for 5 min at 4 °C. Nuclei were pelleted at 1000 × g, and the resulting supernatant was layered on a 10–35% (w/v) linear sucrose gradient (4 ml), with a 50% cushion (0.75 ml), in gradient buffer (10 mM Tris (pH 8.0), 50 mM potassium acetate, 1 mM magnesium acetate, 1 mM DTT). Gradients were centrifuged at 35,000 rpm for 3 h at 5 °C with a Beckman SW55 Ti rotor. 250-μl fractions were collected with an Isco model 185 density gradient fractionator connected to a Pharmacia UV monitor and fraction collector. Collected fractions were treated with protease K (0.2 mg/ml) in 0.2% SDS, 42 °C for 20 min, phenol-extracted, and ethanol-precipitated. 5 to 10 μg of tRNA was added to each fraction prior to precipitation. Precipitated RNA was applied to a 1.0% denaturing agarose gel and analyzed by standard ethidium bromide staining and Northern blotting techniques. Fractions 1 and 2 and 3 and 4 were combined for gel analysis.

**RESULTS AND DISCUSSION**

Scrambled control oligonucleotides were tested in a dose-response analysis to verify that inhibition of ICAM-1 protein expression by the 2′-O-(2-methoxy)ethyl-modified oligonucle-
ment with most active RNase H-compatible antisense oligonucleotides which complement the 5’ cap region of ICAM-1. Northern analysis of total cellular RNA. A, Northern blots for ICAM-1 and G3PDH. Each oligonucleotide was tested at a concentration of 50 nm. Cells were harvested at 4 and 20 h post 1-h induction by TNF-α. ISIS 11929 is a phosphodiester 2’-O-(2-methoxy)ethyl-modified anti-E-selectin oligonucleotide used as a control in this experiment.

To determine whether the antisense effect on transcript abundance was specific to ICAM-1, blots were probed for the E-selectin transcript, normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA (G3PDH). Each oligonucleotide was tested at a concentration of 50 nm. Cells were harvested at 4 and 20 h post full-time induction by TNF-α. ISIS 11929, 5’-GAAGTCAGC-CAAGAACAGCT-3’, is complementary to nucleotides 1–20 of the E-selectin transcript (47).

FIG. 4. Increase in target transcript abundance occurs in cells treated with 2’-O-(2-methoxy)ethyl-modified antisense oligonucleotides which complement the 5’ cap region of ICAM-1. Northern analysis of total cellular RNA. A, Northern blots for ICAM-1 and G3PDH. B, bar graph showing relative abundance of ICAM-1 transcript, normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA (G3PDH). Each oligonucleotide was tested at a concentration of 50 nm. Cells were harvested at 4 and 20 h post 1-h induction by TNF-α. ISIS 11929 is a phosphodiester 2’-O-(2-methoxy)ethyl-modified anti-E-selectin oligonucleotide used as a control in this experiment.

FIG. 5. Increase in target transcript abundance also occurs in cells treated with 2’-O-(2-methoxy)ethyl-modified antisense oligonucleotides which target the 5’ cap region of E-selectin. Northern analysis of total cellular RNA. A, Northern blots for E-selectin and G3PDH. B, bar graph showing relative abundance of E-selectin transcript, normalized to the G3PDH transcript. Each oligonucleotide was tested at a concentration of 50 nm. Cells were harvested at 4 and 20 h post full-time induction by TNF-α. ISIS 11929, 5’-GAAGTCAGC-CAAGAACAGCT-3’, is complementary to nucleotides 1–20 of the E-selectin transcript (47).

FIG. 6. Nuclear and cytosolic fractionation was utilized to determine if antisense inhibition of ICAM-1 protein expression resulted from inhibition of transport of the mature transcript out of the nucleus to the cytoplasm. Fractionated mRNA was evaluated by Northern analysis 2 h post TNF-α induction for 2’-O-(2-methoxy)ethyl oligonucleotide-treated (phosphorothioate and phosphodiester; antisense and control each at 50 nM) and untreated cells (Fig. 6). At this time point no substantial alteration in the abundance of the ICAM-1 transcript was observed in the nuclear fractions of antisense treated cells (110–114%) versus scrambled control treated (113–118%) and untreated (100%). In contrast, a significant increase in the abundance of the ICAM-1 transcript was observed in the cytosolic fraction from the antisense treated cells, ISIS 11158 (230%) and ISIS
11159 (181%), in comparison to the scrambled control treated, ISIS 12344 (133%) and ISIS 12345 (108%), and untreated cells (100%). Relative abundance of the ICAM-1 transcript in each compartment was also determined 4 h post 1-h TNF-α induction. Under these conditions, the relative abundance of the ICAM-1 transcript was 451 and 425% in the cytosolic fraction and 128 and 126% in the nuclear fractions of the antisense treated cells, ISIS 11158 and 11159, respectively, relative to the untreated cells. The significant increase in abundance of the transcript in the cytoplasm of the antisense treated cells suggested a decrease in the rate at which the transcript is normally degraded. The lack of a substantial change in ICAM-1 mRNA abundance in the nuclear fraction indicated that the antisense oligonucleotides did not significantly affect the nucleocytoplasmic transport rate of the mature ICAM-1 transcript.

Polysome profiles were utilized to determine the effect of antisense oligonucleotide treatment upon the translation process of the target ICAM-1 transcript (Fig. 7). ICAM-1 protein and mRNA were evaluated 4 h after a 1-h TNF-α induction from cells treated with antisense oligonucleotides ISIS 11158 and 11159, and their respective scrambled controls ISIS 12344 and 12345. Cytosolic extracts were sedimented by linear sucrose gradient centrifugation (10–35%). The ethidium bromide-stained gel of the fractionated RNA showed a respectable separation of the subpolysomal and polysomal pools (Fig. 7A). Assignment of the fractions were verified by UV absorbance plots obtained during fractionation. Northern blots showed a significant difference in the polysomal distribution of the ICAM-1 transcript in cells treated with ISIS 11158 and 11159, in comparison to those of the controls, ISIS 12344 and 12345 (Fig. 7B). The polysome profiles for the ISIS 11158 and 11159 treated cells showed the majority (71 and 65%, respectively) of the full-length target transcript localized in the subpolysome fractions, e.g. 40 s and 60 s, whereas the ISIS 12344 and 12345 polysome profiles showed the majority (77 and 86%, respectively) of the target transcript in the monosome and polysome fractions.

The polysome profile data for ISIS 11158 and 11159 indicate that inhibition of ICAM-1 protein expression occurs through interference with translation initiation and specifically ribosomal assembly, as indicated by the dramatic redistribution of transcript into the subpolysomes. The formation of a stable antisense-mRNA duplex (or secondary structure) in the 5’ cap region is likely the basis of this effect (see Table I). The increase in abundance of the ICAM-1 mRNA in the cytosolic fraction of the antisense treated cells in conjunction with the change in the polysome distribution patterns indicates that one of the target transcript’s decay pathways is coupled to translation. These data are consistent with observations of transcripts that contain stability determinants in the coding region, e.g. c-fos and c-myc (50, 51).

Regulation of gene expression may occur at one or more stages of mRNA metabolism. The most well known examples of regulation through mRNA metabolism have been found at the stages of translation (52) and degradation (53) of the mature transcript, where certain mRNA sequences and structural elements have been found to be key regulatory determinants. Of particular relevance, it has been shown that stable secondary and tertiary structures located in the 5’-terminal region may regulate initiation of translation (54–56). The 2’-O-(2-methoxyethyl-modified antisense oligonucleotides, complementary to the 5’-terminal region of the target transcript (nucleotides 1–20), mimic this endogenous mode of regulation in cells
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by inhibiting formation of the 80 S translation initiation complex. We believe that this event in turn affects the turnover rate of the transiently expressed ICAM-1 transcript.

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