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 molecular 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 targeting 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 substituents 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 transcription led to the selection of the exceptionally active 2'-O-(2-methoxy)ethyl-modified oligonucleotides, 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 (Received for publication, November 19, 1996)
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**A ICAM-1 mRNA 5′ UTR Sequence**

5′ m7GpppGAGCCUCUCUGCAUG. 3′

**B Antisense Oligonucleotide Sequence and Structures**

5′ TCTGAGTAGCAGGAGCTC 3′

FIG. 1. A, 5′-UTR sequence of the ICAM-1 mRNA derived from normal HUVECs induced by TNF-α (18). The antisense binding region is underlined. B, antisense oligonucleotide sequence and structures. Antisense oligonucleotides are complementary to nucleotides 1–20 of the ICAM-1 mRNA. The 3′-terminal residue of each oligonucleotide was a 2′-deoxy. 2′-O-(2-Methoxy)ethyl-modified cytosines were methylated at the 5 position, e.g., 5-methylcytosine. 2′-O-Methyl, 2′-O-allyl, and 2′-fluoro modified oligonucleotides contained uridine in lieu of thymine.

**Table I**

**Biological and biophysical profiles of modified antisense oligonucleotides**

| ISIS number | 2′-Sugar modification | Antisense activity | Duplex stability |
|-------------|------------------------|--------------------|-----------------|
|             |                        | Rank | IC50 | Tm (°C) | ΔTm | ΔΔG° (°C) |
| PO          | -O-(2-Methoxy)ethyl    | 1    | 2.1  | 87.1    | 37.0 | -9.14     |
| 11158       | -O-Allyl               | NA   | 76.5 | 26.4    | -7.37 |
| 12461       | -O-Methyl              | NA   | 83.7 | 33.6    | -7.85 |
| 3214        | -H                     | NA   | 58.4 | 8.3     | -2.8  |
| 3061        |                        |      |      |         |       |
| PS          | -O-(2-Methoxy)ethyl    | 2    | 6.5  | 79.2    | 29.1 | -6.95     |
| 11159       | -F                     | 3    | 25   | 87.9    | 37.8 | -8.47     |
| 11665       | -O-Allyl               | 4    | 34   | 70.3    | 20.2 | -5.75     |
| 12462       | -O-Methyl              | 5    | 41   | 50.1    |      | Parent    |
| 3067        | -H                     | 6    | ≥50  | 76.4    | 24.3 | -6.16     |
| 3224        | -O-Methyl              |      |      |         |       |

**Table**

*Antisense activity (IC50) was determined by measurement of cell surface expression of ICAM-1 protein following treatment with each oligonucleotide at six concentrations in the range of 1.6–50 nM, as described under "Experimental Procedures." IC50 values were calculated from the average of two sets of dose-response data points, where NA indicates that no IC50 was achieved or observed in the given dose range. Thermal melt analysis was performed as described previously (23), where**

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 times with Opti-MEM (Life Technologies, Inc.) in Opti-MEM, serially diluted to the desired concentration 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 GRP centrifuge), resuspended in PBS, and stained with 3 μl/10^7 cells of the ICAM-1 specific antibody, CD54-PE (Becton Dickinson) and 0.1 μg of the control antibody, IgG2b-PE (Pharmingin). 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 FACSscan. Percentage of the control ICAM-1 expression was calculated as follows: (oligonucleotide-treated ICAM-1 value) – (basal ICAM-1 value) / (non-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 G3PDH. The 6X174 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 Catrimox-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 (Amer sham). Blots were hybridized with Prime-a-Gene cDNA probes (Promega) using RapidHyb (Amer sham). 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 (pHcGAP, 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, 5 mM mercaptoethanol, phenol-extracted under acidic conditions (pH 4.0), and isopro pyl alcohol-precipitated (41). The cytosol precipitate was resuspended in 300 µl of the denaturing solution plus 30 µl of 2× sodium acetate (pH 4.0) and reprecipitated with isopro pyl alcohol. 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 106 oligonucleotide-treated cells were pelleted, washed with PBS, 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 proteinase K (0.2 mg/ml) in 0.2% SDS at 42 °C for 20 min, phenol-extracted, and ethanol-preci patitated. 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 oligonucleo-

tides, ISIS 11158 and 11159, was sequence-specific. The respective scrambled control oligonucleotides, ISIS 12344 and 12345, showed negligible effects on ICAM-1 protein expression (Fig. 2). As indicated in Table I, both the phoshodiester, ISIS 11158, and the phosphorothioate, ISIS 11159, 2′-O-(2-methoxy)ethyl-modified oligonucleotides were more potent inhibitors of ICAM-1 expression in HUVECs than the analogous RNase H-compatible phosphorothioate oligodeoxynucleotide, ISIS 3067.

Intracellular distribution of the 2′-O-(2-methoxy)ethyl-modified oligonucleotides was determined by fluorescence microscopy, using fluorescein-labeled oligonucleotides, to further compare and delineate the basis of their antisense activity with respect to the first generation 2′-deoxyoligonucleotides (Fig. 3). As reported previously (42) treatment of HUVECs with the fluorescein-labeled 2′-deoxy phosphorothioate oligonucleotide, in the presence of the cationic lipid formulation (Lipofectin, Life Technologies, Inc.), resulted in a heterogeneous accumulation of the oligonucleotide in the cell nucleus as well as in punctate cytoplasmic vesicles (Fig. 3A). Treatment of HUVECs with the 2′-deoxy phoshodiester analog showed a diffuse distribution of label in the cytoplasm (Fig. 3B), attributed to degradation of the oligomer by nucleases (43, 44). In comparison, the fluorescein-labeled 2′-O-(2-methoxy)ethyl-modified phosphorothioate oligonucleotide yielded a distribution pattern (Fig. 3C) similar to the 2′-deoxy analog (Fig. 3A), with a high degree of localization in the nucleus. However in striking contrast to the 2′-deoxy analog (Fig. 3B), the 2′-O-(2-methoxy)ethyl phosphodiester showed a homogeneous nuclear localization (Fig. 3D), attributed to its greater resistance to nucleases, the absence of “nonspecific” phosphorothioate-protein interactions (45, 46), and possibly more compatible interactions with the lipid formulation for uptake and delivery.

Total cellular RNA was isolated and analyzed to determine whether inhibition of ICAM-1 protein expression by ISIS 11158 and 11159 resulted from antisense-promoted degradation of the target transcript, an end point observed following treat-
ment with most active RNase H-compatible antisense oligonucleotides (4, 5). Total RNA was harvested at 4 and 20 h following a 1-h TNF-α induction period for untreated and oligonucleotide-treated HUVECs. Interestingly, Northern blot analysis showed a significant increase in relative abundance of the ICAM-1 transcript in those cells treated with the anti-ICAM-1 oligonucleotides at both time points (Fig. 4). Nuclear runoff experiments showed that this increase in transcript abundance was not due to an increase in the rate of transcription of the ICAM-1 gene (data not shown).

To determine whether the antisense effect on transcript abundance was specific to ICAM-1, blots were probed for the E-selectin transcript whose expression is also transiently induced by TNF-α in HUVECs. As with ICAM-1 an increase in abundance of the E-selectin transcript was observed only in those cells treated with the 2’-O-(2-methoxy)ethyl-modified oligonucleotide, ISIS 11929, that targets the 5’-terminal region of the E-selectin oligonucleotide used as a control in this experiment.

The 5’ cap structure of mRNA has been shown to facilitate splicing of the first intron of pre-mRNA constructs in several different systems (48, 49). The ICAM-1 gene consists of seven exons separated by six introns, with intron 1 approximately 4000 nucleotides in length (16). Evaluation of the Northern blots showed that this set of modified antisense oligonucleotides had no effect on splicing of the ICAM-1 pre-mRNA to the mature transcript, as evidenced by lack of ICAM-1 mRNA species or intermediates of longer lengths (data not shown).

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 11929.

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-CAGAAGACAGCT-3’, is complementary to nucleotides 1–20 of the E-selectin transcript (47).
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 subpolysome fractions. 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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FIG. 7. Inhibition of translation initiation proves to be key mode of action for 2‘-O-(2-methoxy)ethyl-modified oligonucleotides that target the 5’ cap region of the ICAM-1 transcript in HUVECs. Polyacrylamide distribution profiles for cells treated with ISIS 12344 (phosphodiester scramble control), ISIS 11159 (phosphodiester antisense), ISIS 12345 (phosphorothioate scramble control), and 11159 (phosphorothioate antisense), each at 50 nM, ethidium bromide-stained agarose gels; B, Northern blots. Oligonucleotide-treated cells were harvested 4 h post TNF-α induction (1-h period) as described. A total RNA standard (fraction S) was included on each gel for blot to blot normalization.
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