

Oligonucleotides Antisense to the Interleukin 1 Receptor mRNA Block the Effects of Interleukin 1 in Cultured Murine and Human Fibroblasts and in Mice

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Abstract

Phosphodiester and phosphorothioate oligodeoxynucleotides (18 mers) were constructed antisense to sequences of the recently cloned murine and human IL-1 receptors. Murine antisense oligonucleotides inhibited IL-1-stimulated PGE₂ synthesis by murine fibroblasts in culture in a time (days) and concentration-dependent (3 μ M–30 μ M) fashion. Murine sense oligonucleotide and an oligonucleotide antisense to human IL-1 receptor were without effect. Moreover, murine antisense oligonucleotides did not affect tumor necrosis factor- or bradykinin-stimulated PGE₂ synthesis by murine fibroblasts. Similarly, antisense oligonucleotides to the human, but not the murine, IL-1 receptor inhibited IL-1-stimulated PGE₂ synthesis by cultured human fibroblasts. The attenuation of the cellular response to IL-1 caused by the antisense oligonucleotides correlated with a loss in cell surface receptors for IL-1, without any change in the number of bradykinin receptors on these cells. When antisense oligonucleotides were encapsulated in liposomes, they blocked completely the appearance of newly synthesized IL-1 receptors and IL-1-stimulated PGE₂ synthesis. In mice, subcutaneous injection with an oligonucleotide antisense to the murine IL-1 receptor markedly inhibited the infiltration of neutrophils in response to subsequent injection of IL-1. These data suggest that antisense oligodeoxynucleotides may share a role in the design of antiinflammatory therapeutics. (*J. Clin. Invest.* 1991. 88:1190–1196.) Key words: inflammation • dermatitis • neutrophil infiltration

Introduction

IL-1, a cytokine released from a variety of cells in response to inflammatory insult, is implicated in the pathogenesis of several chronic inflammatory diseases, including rheumatoid arthritis (1). IL-1 elicits its effects by binding to specific cell-surface receptors on a broad spectrum of cell types. IL-1 receptors expressed by T lymphocytes, endothelial cells, and fibroblast-like cells (so called type I receptors) are thought to be identical, whereas IL-1 receptors on macrophages and B lymphocytes (type II receptors) are different (2–4). Recently, type I IL-1 receptors have been cloned from human and murine T lymphocyte and human fibroblasts (5–7) and the amino acid sequences were found to be highly conserved between species.

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We wish to study the expression and activation of IL-1 receptors in cells and to assess the role of IL-1 in physiological and pathological processes. Evidence that IL-1 is implicated in the pathogenesis of certain inflammatory diseases has generated great interest in developing therapeutic agents which inhibit the biological effects of this cytokine. Antisense oligodeoxynucleotides present a potential alternative therapeutic approach. Antisense oligodeoxynucleotides have been used to block translation of proteins both in cell-free translation systems and in cultured cells (8). Use of natural phosphodiester-containing oligonucleotides (O¹-oligos) in studies using intact cells or animals is limited by their rapid metabolism by endogenous nucleases (9). However, phosphorothioate oligonucleotides (S-oligos) are more nuclease-resistant (10), making them potentially useful in vivo, even though they are taken up into cells more slowly (9). To date, however, most studies using antisense oligodeoxynucleotides have focused on the inhibition of expression of viral genes and oncogenes. To assess the capability that these agents may possess to modulate cytokine-mediated responses, we have designed species-specific oligodeoxynucleotides antisense to mRNA sequences for the human and murine IL-1 receptors. We have used these agents to study aspects of cellular and species-specific targeting and their ability to attenuate IL-1 mediated responses in vitro. In addition, we have extended these findings to an in vivo model of cytokine-mediated inflammation to block neutrophil infiltration in response to local administration of IL-1 in mouse dermal tissue.

Methods

Cell culture. 3T3 fibroblasts were cultured as described in DMEM supplemented with 10% calf serum (11) to confluency in 96-well plates. Human dermal fibroblasts were obtained from Clonetics Corp., San Diego, CA, and were cultured in DMEM containing 10% fetal bovine serum.

IL-1 binding. IL-1 binding experiments were performed as described (12). Confluent cultures in 24-well plates were washed twice with HBSS containing 20 mM Hepes, pH 7. To each dish was added 200 μ l ice-cold HBSS containing 20 mM Hepes, 1 mg/ml BSA, and 50 nCi human recombinant [¹²⁵I]IL-1 α (New England Nuclear, Boston, MA). For Scatchard analyses, unlabeled IL-1 α (Boehringer Mannheim Corp., Indianapolis, IN) was added to vary concentration. Nonspecific binding was determined in the presence of 2 nM IL-1 β (Boehringer Mannheim). To terminate experiments, the cultures were incubated on ice for 60 min, then each culture was washed four times with 10 ml ice-cold HBSS containing 1 mg/ml BSA. Cells were removed from plates using HBSS containing trypsin. Cell-associated radioactivity was determined using a gamma counter and was ~ 3,000 dpm/well in the absence of added unlabeled IL-1 and 100–150 dpm in the presence of 2

1. Abbreviations used in this paper: h, human; m, murine; O, phosphodiester; S, phosphorothioate.

nM unlabeled IL-1 β . K_d and maximal binding capacity (B_{max}) were determined using LIGAND (Biosoft, Milltown, NJ) (13).

Bradykinin binding. Bradykinin receptor binding to cell membranes was carried out as described (11). Cells were scraped into 100 vol of a buffer composed of 25 mM N-tris(hydroxymethyl)methyl-2-aminooethane-sulfonic acid, 0.2 g/liter 1,10-phenanthroline, pH 6.8, and homogenized using a Polytron Tisumizer (Brinkmann Instruments, Inc., Westbury, NY) at setting 6 for 15 s. The homogenate was centrifuged at 50,000 g for 10 min, the supernatant discarded, and the pellet resuspended into the same buffer also containing 1 g/liter BSA and 0.14 g/liter bacitracin. To assay tubes were added [3 H]bradykinin, the amount of radioactivity being varied in the tubes to vary concentration for Scatchard analysis. Nonspecific binding was determined in the presence of 10 μ M NPC 567, a bradykinin antagonist (11). Assays were terminated by filtration through glass fiber filters that had been soaked in 2 g/liter polyethyleneimine for 1 h, and washed four times with 4 ml of ice-cold 50 mM Tris, pH 7.4.

PGE₂ synthesis. PGE₂ was quantitated from direct aliquots of media using a radioimmunoassay as previously described for these cells and media (11). Reagents were from Advanced Magnetics, Inc., Cambridge, MA, and control experiments demonstrated that none of the experimental agents (IL-1, tumor necrosis factor, bradykinin, or antisense oligonucleotides) affected the assay.

Liposomes. Liposomes were constructed of phosphatidylserine (14), loaded with oligonucleotides, and fused to the cells (9). Phosphatidylserine (Avanti Polar Lipids, Inc., Birmingham, AL), 5 mg in 1 ml chloroform, was placed into a spherical flask and, under a nitrogen atmosphere, the chloroform was removed using a rotary evaporator, leaving a film of lipid. The lipid was resuspended in 0.2 ml EDTA buffer (100 mM NaCl, 2 mM histidine, 2 mM Tris acid, 0.1 mM EDTA, pH 7.4) with vigorous vortexing to form multilamellar vesicles. Then CaCl₂ was added to 10 mM to form cochleate bodies. Oligonucleotide was added and the mixtures were allowed to stand at room temperature for 1 h. EDTA was then added to 15 mM and the pH adjusted to 7 to cause liposome formation. This solution was allowed to stand for 30 min. Liposomes were concentrated by centrifugation at 100,000 g for 30 min at room temperature and gently washed with HBSS. For lipofusion, cells were rinsed with HBSS, then incubated in HBSS containing 2 mM calcium and 0.1 mM magnesium for 30 min. Liposomes were added to a concentration of 1.25 mg/ml lipid and incubated for 30 min. To the cultures was then added a volume of polyethylene glycol (6,000 average molecular mass) equal to the salt solution in the well (resulting in 50% polyethylene glycol) for 1 min, followed by addition of culture medium containing serum, and careful washing of the wells three times to remove the polyethylene glycol. In an experiment to determine the efficiency of oligonucleotide incorporation, oligonucleotide was 32 P-end-labeled before incorporation into liposomes. After one wash 12% of radioactivity was retained in the liposome pellets. Re-

peated washing demonstrated that 6–7% of radioactivity was stably retained.

Labeling neutrophils. In the experiments in mice, in vivo, the day before IL-1 was to be administered, animals received 10 μ Ci [3 H]thymidine i.p. to label circulating neutrophils. Using this method, 24 h after injection, virtually all radioactivity in the blood is contained within the DNA of neutrophils (15). Hair was shaved from the backs before the beginning of the experiment. IL-1 was administered intradermally 1 cm lateral to the spine and, 4 h later, animals were killed by cervical dislocation. Skin was removed and 8 mm punch biopsies were obtained. These were dissolved in Protosol (New England Nuclear) overnight, then radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by taking biopsies from areas that had been injected intradermally with saline not containing IL-1. Each animal served as its own control in that murine antisense oligonucleotides were injected on one side of the midline whereas on the other side, injections contained the human antisense oligonucleotide, which is inactive in mouse cells in culture (see *Results*).

Oligonucleotides. Oligodeoxynucleotides were synthesized (Synthecell Corp., Rockville, MD) complementary to unique species-specific coding regions of mRNA for murine and human IL-1 receptors. These included 18-base regions at the initiation codon (mO-oligo-1, 5'-CACTTTCATATTCTCCAT, complementary to bases –57 to –40 of the murine sequence [6], and mS-oligo-1, the analogous phosphorothioate analog; hO-oligo-1, 5'-TCTGAGTAACACTTTCAT, complementary to bases –51 to –34 of the human IL-1 receptor sequence [5], and hS-oligo-1, the analogous phosphorothioate analogue), an internal open reading frame site (mS-oligo-2, a phosphorothioate analogue of sequence 5'-GAGACAAATGAGCCCCAG, complementary to bases –36 to –19 of the murine sequence), and a region immediately 5' to the termination codon (mS-oligo-3, a phosphorothioate analogue of sequence 5'-GCCGAGTGGTAAGTGTGT complementary to bases 1654–1671 of the murine sequence). Two sense phosphorothioate oligonucleotides were used, equivalent to mS-oligo-1, 5'-ATGGAGAA-TATGAAAGTG, and hS-oligo-1, 5'-ATGAAAGTGTACTCAGA as controls.

Results

Time course for inhibition of IL-1-stimulated PGE₂ synthesis by antisense oligonucleotides. In initial experiments, both mO-oligo-1 and mS-oligo-1, 10 μ M, inhibited IL-1-stimulated PGE₂ synthesis in cultures of murine fibroblasts grown in media with serum that had been heat-inactivated to denature nucleases (16) (Fig. 1 A). The O-oligo was more acute in effect,

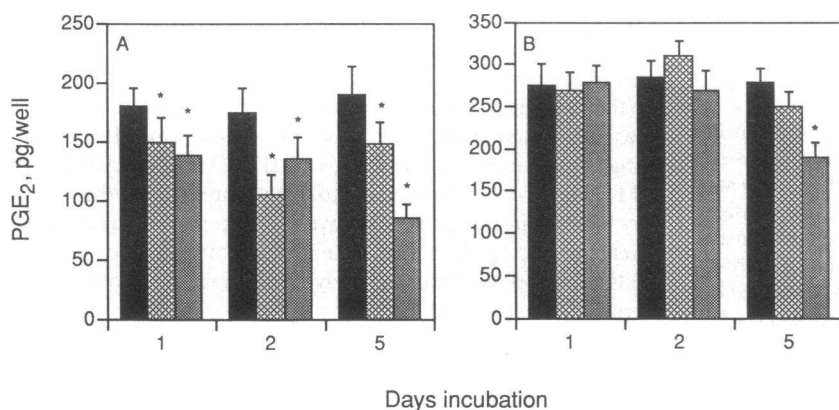


Figure 1. Time course for inhibition of IL-1-stimulated PGE₂ synthesis by antisense oligonucleotides in murine fibroblasts. 3T3 fibroblasts were cultured to confluency, then 10 μ M mO-oligo-1 or mS-oligo-1 was added. After incubation for the indicated times, the media were replaced and IL-1 α , 100 U/ml was added, and cultures were incubated for 4 h. Then media were collected and assayed for PGE₂. IL-1 stimulation in control cultures (filled bars), and in cultures incubated with mO-oligo-1 (crossed bars) or mS-oligo-1 (stippled bars). Basal synthesis was 42–56 pg/well. * P < 0.05 compared to no oligonucleotide by Student's t test for paired data. (A) Cultures contained 10% heat-inactivated calf serum. (B) Cultures contained 10% calf serum that had not been heat inactivated. Data are mean \pm SEM for triplicate cultures.

while the effect of the S-oligo became more pronounced over a longer period of time. In culture media containing serum that had not been heat-inactivated the O-oligo was without effect, while the S-oligo was still capable of some inhibition of the biological effect of IL-1 (Fig. 1 B).

Concentration response for inhibition of IL-1-stimulated PGE₂ synthesis by antisense oligonucleotides. To compare the relative potencies of several antisense oligonucleotides directed to different regions of mRNA for IL-1 receptors, murine fibroblasts were incubated with various concentrations of S-oligos for 48 h. mS-oligo-1, a sequence antisense to the initiation site, was more potent at inhibiting IL-1-stimulated PGE₂ synthesis than mS-oligo-2, which is antisense to a sequence immediately downstream from mS-oligo-1 (Fig. 2 A). At a concentration as low as 3 μ M, mS-oligo-1 significantly inhibited IL-1-stimulated PGE₂ synthesis. mS-oligo-3, which is antisense to a sequence near the termination codon of the IL-1 receptor mRNA, was without effect on IL-1-stimulated PGE₂ synthesis.

Inhibition of IL-1 receptor-mediated PGE₂ synthesis by the antisense oligonucleotides was selective. While mS-oligo-1 blocked IL-1-stimulated PGE₂ synthesis, it was without effect on tumor necrosis factor- or bradykinin-induced PGE₂ synthesis. Tumor necrosis factor (Genzyme Corp., Cambridge, MA), 10 nM, stimulated PGE₂ synthesis from 45 ± 6 to 216 ± 15 pg/well in 4 h in control cultures, and from 38 ± 10 to 210 ± 18 pg/well in cultures that had been incubated with 30 μ M mS-oligo-1 for 2 d. Bradykinin, 1 μ M, stimulated PGE₂ synthesis from 12 ± 3 to 44 ± 6 pg/well in 5 min, and from 15 ± 5 to 47 ± 5 pg/well in cultures incubated with 30 μ M mS-oligo-1 for 2 d. In addition, neither hS-oligo-1, an antisense oligonucleotide directed to the initiation codon region of the human IL-1 receptor, nor the sense oligonucleotide complementary to mS-oligo-1, had any effect on IL-1-stimulated PGE₂ synthesis in murine fibroblasts (Fig. 2 and data not shown). Analogous to murine fibroblasts, the response of human dermal fibroblasts to IL-1 was inhibited by incubation with hS-oligo-1, but not by mS-oligo-1 or the sense oligonucleotide complementary to hS-oligo-1 (Fig. 2 B and data not shown).

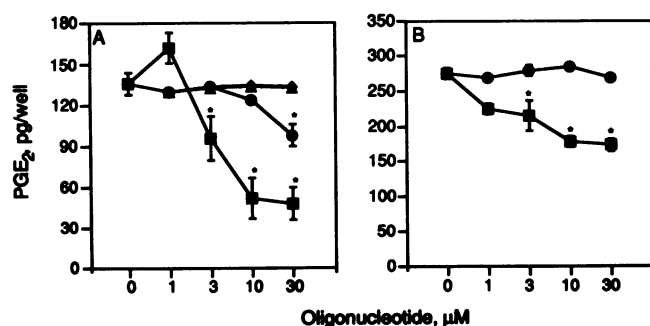


Figure 2. Concentration response for inhibition of IL-1-stimulated PGE₂ synthesis by oligodeoxynucleotides in murine and human dermal fibroblasts. Confluent cultures were incubated with the indicated S-oligos for 48 h, then media were replaced and IL-1, 100 U/ml, was added and cultures were incubated for 4 h before collection of media for assay of PGE₂. (A) IL-1-stimulated PGE₂ synthesis in 3T3 fibroblasts in the presence of mS-oligo-1 (■), mS-oligo-2 (●), mS-oligo-3 (▲), or hS-oligo-1 (◆). Data are mean \pm SEM for triplicate cultures. (B) IL-1-stimulated PGE₂ synthesis in human dermal fibroblasts in the presence of hS-oligo-1 (■) or mS-oligo-1 (●). Data are mean \pm SEM for triplicate cultures. * $P < 0.05$ compared to no oligonucleotide.

Antisense oligonucleotides reduce expression of IL-1 surface receptors. The attenuation of the cellular response to IL-1 caused by the antisense oligonucleotides correlated with a loss in cell surface receptors for IL-1 in murine fibroblasts. The number and affinity of IL-1 receptors were assessed in murine fibroblasts treated with mS-oligo-1 for 48 h and compared to control fibroblasts. Scatchard analysis demonstrated that the affinity of IL-1 receptors was not altered by treatment with mS-oligo-1, but receptor number was decreased to only 55% of control (Fig. 3). In contrast, mS-oligo-1 did not affect the number of receptors for bradykinin (Fig. 4), demonstrating that the effects of the antisense oligonucleotides were specific to the IL-1 receptor and not due to a cytotoxic effect on the cells.

Encapsulation in liposomes enhances the activity of S-oligos. The time course over which antisense oligonucleotides inhibited cellular responsiveness to IL-1 may have reflected slow turnover of receptors at the cell surface or slow uptake of the oligonucleotides, especially the S-oligos, into cells. To gain insight into which mechanism was predominant, we enhanced delivery of hS-oligo-1 by liposome encapsulation and fusion to human dermal fibroblasts under conditions of steady-state (basal) turnover of receptors and in cells undergoing rapid turnover of their IL-1 receptors. Thus, in the latter case, we treated human fibroblasts for 18 h with 1 nM IL-1 to downregulate IL-1 receptors and IL-1 was then removed to allow recovery of receptors via new receptor synthesis (17–19). We included hS-oligo-1 free in solution or encapsulated in liposomes, to determine whether liposomal delivery of the oligonucleotide into cells could block recovery of responsiveness to IL-1. In cells that had not been exposed to IL-1 to initiate receptor turnover, these short-term incubations of cultures with either free hS-oligo-1 or mS-oligo-1 did not affect response to IL-1 as would be predicted from the data in Fig. 1. Of interest, exposure of cells during steady-state turnover of IL-1 receptors to liposome-encapsulated hS-oligo-1 was also without short-term effect on response to IL-1 (Table I). If, however, cells were exposed to liposome-encapsulated hS-oligo-1 during a period of new receptor expression, complete blockade of the ability of cells to recover responsiveness to activation of IL-1 receptors was observed. No effect of hS-oligo-1 free in solution was seen under these conditions.

Parallel with the studies of recovery of IL-1 responsiveness in the downregulated fibroblasts, we performed IL-1 receptor binding experiments to assure that receptor number correlated with biological responsiveness. Receptor binding studies were carried out as in Fig. 3, but monitoring only specific binding as described (18). Thus, radiolabeled IL-1 (10 nCi) was added to each well in the absence or presence of 2 nM unlabeled IL-1 to account for nonspecific binding. Total specific binding was defined as binding in the presence of radiolabeled IL-1 alone minus binding in the presence of 2 nM unlabeled IL-1. Similar to the biological response, incubation of cells with IL-1 for 18 h was associated with a decrease in receptor number which largely recovered 4 h after removing IL-1 (Table II). Adding 30 μ M hS-oligo-1 free in solution to the cultures during the recovery period did not prevent recovery of receptor number, however, enhancing the delivery of the oligonucleotide into the cells using liposomes completely blocked recovery of IL-1 receptors: in cells to which empty liposomes had been fused at the beginning of recovery time, specific binding returned to control levels, while in cells that had been fused with liposomes containing a final concentration of 30 μ M hS-oligo-1, specific

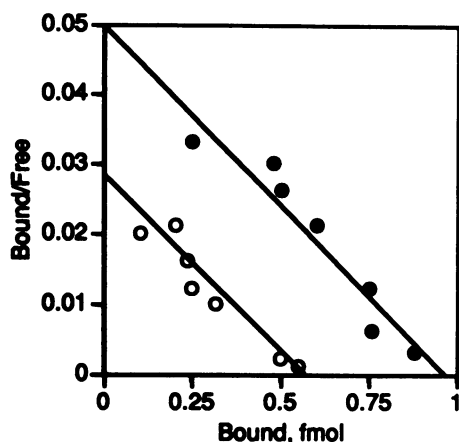


Figure 3. Scatchard analysis of binding of human recombinant IL-1 α to cell surface receptors on 3T3 fibroblasts. Illustrated are results from a single experiment. K_d and B_{max} (from three separate experiments) were 145 ± 26 pM and 1.01 ± 0.09 fmol (2,678 receptors/cell) in control cultures (\bullet) and 132 ± 30 pM and 0.56 ± 0.14 fmol in cultures incubated with mS-oligo-1 ($30 \mu\text{M}$ for 2 d) (\circ). In cultures incubated with hS-oligo-1, $30 \mu\text{M}$, for 2 d, K_d was 165 ± 20 pM and B_{max} was 1.06 ± 0.19 fmol.

binding was unchanged from downregulated levels. Thus the half-life of IL-1 receptors at the cell surface play a major role in the biological efficacy of antisense oligonucleotides although attenuation of expression of receptors is considerably augmented using liposome delivery under conditions of new receptor synthesis.

Antisense oligonucleotides block IL-1 effects in mice. Additional experiments were performed to determine whether the effects of the antisense oligonucleotides on IL-1 receptor function in cultured cells could be extended in vivo to an animal model of cytokine-mediated inflammatory response. Subcutaneous injection of IL-1 in mice causes neutrophil infiltration at the site of injection (20, 21). Mice were injected subcutaneously with either mS-oligo-1 or hS-oligo-1 at 24-h intervals over a period of 3 d. 24 h before subsequent subcutaneous

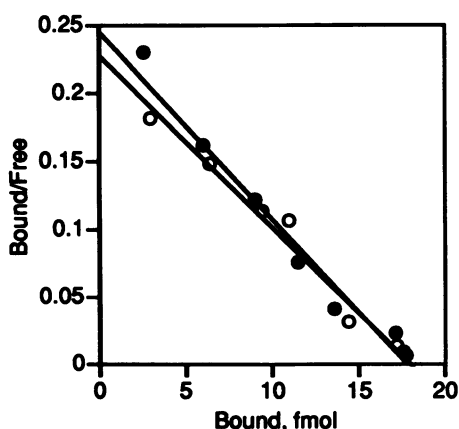


Figure 4. Scatchard analysis of binding of bradykinin to membranes from 3T3 fibroblasts. Illustrated are the results from a single experiment. In membranes from control cultures (\bullet) ($n = 3$) there were $2,960 \pm 410$ receptors per cell with K_d of 112 ± 16 pM compared to $3,045 \pm 360$ receptors per cell with K_d of 125 ± 20 pM in membranes from cells incubated with $30 \mu\text{M}$ mS-oligo-1 (\circ) for 2 d.

Table I. Liposome Delivery of S-oligos to Human Dermal Fibroblasts IL-1-induced PGE₂ Synthesis

Condition	PGE ₂ pg/well
Basal receptor turnover	
Basal	46 \pm 10
IL-1 18 h, wash 8 h	52 \pm 12
no IL-1, wash with media 8 h, IL-1 4 h	744 \pm 22
no IL-1, incubate 8 h with liposome hS-oligo-1, IL-1 4 h	695 \pm 48
no IL-1, incubate 8 h with liposome mS-oligo-1, IL-1 4 h	718 \pm 42
Receptor downregulation/accelerated receptor reappearance	
IL-1 18 h, wash 8 h, IL-1 4 h	398 \pm 25
IL-1 18 h, wash 8 h with hS-oligo-1, IL-1 4 h	385 \pm 32
IL-1 18 h, wash 8 h with mS-oligo-1, IL-1 4 h	376 \pm 36
IL-1 18 h, wash 8 h with liposome hS-oligo-1	68 \pm 15
IL-1 18 h, wash 8 h with liposome mS-oligo-1, IL-1 4 h	285 \pm 40
IL-1 18 h, wash 8 h with liposome hS-oligo-1, IL-1 4 h	74 \pm 25

Data are mean \pm SEM of triplicate wells for each condition. Human dermal fibroblasts grown to confluency in serum-free media (Clonetics Corp.) in 96-well plates were exposed to human recombinant IL-1 (Boehringer Mannheim), 100 U/ml, for 18 h. The cultures were then washed four times with media to remove IL-1 and incubated in IL-1-free media for 8 h. The media contained oligonucleotides, $30 \mu\text{M}$, or liposomes loaded with oligonucleotides, $30 \mu\text{M}$ final concentration in the culture wells, as indicated. At the end of the 8-h incubation period cultures were washed twice and media replaced with culture media containing IL-1 (100 U/ml). Incubation was for 4 h, after which supernatants were collected to quantitate PGE₂. The media in control cultures were changed at the same intervals as the experimental cultures.

injection of IL-1 to initiate neutrophil infiltration, the circulating neutrophil population was labeled with [³H]thymidine (15). Significant reduction of IL-1-stimulated neutrophil infiltration was observed as early as 48 h, or two injections of mS-oligo-1. After three daily injections of mS-oligo-1, IL-1-stimulated neutrophil infiltration was markedly reduced (Table III). In all these experiments, control injections, vehicle or hS-oligo-1, were without effect. Lack of effect of hS-oligo-1, which contains a 5-base mismatch to the murine sequence, correlated well with results obtained in vitro described above.

Discussion

Antisense oligodeoxynucleotides are an attractive potential method to inhibit expression of cell-surface receptors and attenuate cellular responsiveness. Few reports exist that use synthetic oligomers to target receptors in cells. Synthetic antisense RNA to the subunit of the nicotinic acetylcholine receptor inhibited the expression of receptor-activated currents when coinjected with *Torpedo* mRNA in *Xenopus* oocytes (22). Antisense RNA truncated to cover more 5' sequences, however, was less effective than full-length RNA. A single oligodeoxynucleotide directed to 5' sequences also inhibited expression of receptors when it was premixed with *Torpedo* mRNA and coinjected into oocytes. A more recent study found that antisense oligodeoxy-nucleotides directed against the ζ - and β -chains of the

Table II. Liposome Delivery of S-oligos to Human Dermal Fibroblasts: IL-1 Receptor Expression

Condition	Specific Binding of [¹²⁵ I]-IL-1 dpm/well
Basal receptor turnover	
Basal	657±62
Receptor downregulation/acclerated receptor reappearance	
IL-1 18 h, wash	245±30
IL-1 18 h, wash, 4 h recovery	562±46
IL-1 18 h, wash, 4 h recovery with hS-oligo-1	585±60
IL-1 18 h, wash, 4 h recovery with empty liposome	560±38
IL-1 18 h, wash, 4 h recovery with liposome hS-oligo-1	205±16

Data are mean±SEM of triplicate wells for each condition. Human dermal fibroblasts grown to confluency in serum-free media in 96-well plates were exposed to IL-1, 100 U/ml, for 18 h. The cultures were then washed four times with media to remove IL-1 and incubated in IL-1-free media for 4 h. Binding was performed at the times indicated.

murine T cell receptor were effective in suppression of recovery of receptor-mediated function in T cell hybridomas that had been trypsinized to remove existing surface receptors (23).

Since IL-1 receptor-mediated responses are implicated in the pathogenesis of certain inflammatory diseases, we wished to study the effects of antisense oligodeoxynucleotides on the expression and functional activation of IL-1 receptors in cells under conditions more suitable to assess their potential for anti-inflammatory action both in vitro and in vivo.

We have demonstrated that oligodeoxynucleotides antisense to the IL-1 receptor block the expression of IL-1 receptors and the biological effects of IL-1 on cultured cells. The degree to which antisense oligonucleotides inhibited IL-1 stimulated PGE₂ synthesis in cultured fibroblasts varied with several factors. O-oligos, which are sensitive to degradation by nucleases, were inactive in serum-containing medium. In serum, which

Table III. mS-oligo-1 Inhibits IL-1-induced Neutrophil Infiltration in Mice

Condition	Vehicle	mS-oligo-1	hS-oligo-1
		[³ H]Tdr, dpm	
One injection	4658±1135	4425±1238	4534±1286
Two injections	5082±720	3512±410*	4825±602
Three injections	5145±918	3242±484†	4795±1486

* $P = 0.079$, † $P = 0.017$, compared to vehicle, using Student's t test for unpaired observations. Mice received subcutaneous injections of hS-oligo-1 or mS-oligo-1, 3 nmol (25 μ l), or saline, daily into the same sites for one, two, or three injections. The day before IL-1 was to be administered, animals received [³H]thymidine i.p. to label circulating neutrophils (see Methods). Recombinant human IL-1 α , 1,000 U, was injected into each site. After 4 h, animals were killed and punch biopsies were taken. Data are mean±SEM from eight animals. Radioactivity (< 1,000 dpm) in punch biopsies from sites injected with saline alone and not treated with IL-1 have been subtracted from each point.

had been heated to inactivate nucleases, the effect of O-oligos was comparable to nuclease-resistant S-oligos with two distinctions: while the inhibition of IL-1 responsiveness occurred more rapidly, it was also more transient. Our results are consistent with the observations that the specific transport of O-oligos across cell membranes occurs rapidly, in contrast to S-oligos which may have greater affinity for the transporter and are released more slowly into the cell interior (11). Whether the more transient inhibition observed with O-oligos was the consequence of an induction of nuclease activity remains to be determined.

Size is an important consideration in the design of antisense oligonucleotides. In subcellular preparations, it has been found that longer antisense oligonucleotides are more effective than shorter ones, presumably because binding is more thermodynamically favorable between longer complementary strands (8, 24). In studies using intact cells this phenomenon is far less apparent due to the inefficient transport of long oligodeoxynucleotides into cells (22, 25). We chose 18 mers since this size should yield absolute theoretical specificity for a single mRNA (26) while being still short enough to be transported into cells.

In addition to length, target sequences of antisense oligonucleotides play an important role in their biological efficacy. In general, antisense oligonucleotides directed to 5' regions of eucaryotic mRNA, particularly near the initiation codon sequences, are more effective than those directed to more 3' regions (8, 24). While in general agreement, our studies have extended these findings to show that even closely adjacent target sequences yield antisense oligonucleotides with strikingly different potencies. The most inhibitory murine S-oligo (mS-oligo-1; IC₅₀ 3 μ M), directed to the initiation codon, was 10-fold more potent than mS-oligo-2, directed some 20 bases downstream. The low micromolar potency observed in murine fibroblasts agrees well with that observed for S-oligos in a number of studies (8). Moreover, strict species specificity of inhibition was observed even though base mismatches varied from only two to five bases between murine and human IL-1 receptor sequences. Whether the inhibitory effects of antisense oligonucleotides are attributable to the degradation of mRNA by an RNase H-like activity in cells (27) or to translational arrest (28) it seems likely that local secondary structure of mRNAs plays a critical role in the biological efficacy of these agents.

In resting human dermal fibroblasts the antisense oligonucleotide only poorly inhibited IL-1 receptor expression after 48 h incubation. However, delivery of antisense oligonucleotides by encapsulation in liposomes and fusion to cells markedly increases their inhibitory potential, to essentially 100%. In our studies, however, we found this only to be true under conditions where new receptor synthesis predominantly accounted for cellular responsiveness. Thus protein turnover plays an important role in the consideration of the use antisense agents in the inhibition of gene expression. The results obtained in the downregulation study with human dermal fibroblasts are consistent with an 11-h half-life for IL-1 receptors determined using IL-1 binding in cycloheximide-treated T lymphocytes (19).

Evidence that IL-1 plays a major role in the mediation of many chronic inflammatory diseases, including rheumatoid arthritis (1), has generated great interest in developing agents which inhibit the biological effects of this cytokine. Approaches being explored include drugs that block secretion of IL-1 from inflammatory cells (29–31), use of a soluble, recombinant extracellular binding domain of the IL-1 receptor to bind and

deplete circulating cytokine (32), and competitive antagonism of the IL-1 receptor (33–35). Several proteins that compete with IL-1 for its receptor have been cloned (34, 35); at least one is being investigated as a potential therapeutic agent.

Antisense oligonucleotides present an attractive therapeutic approach predominantly due to their nonpeptidic nature and potential to be absolutely specific. Because the effect of IL-1 in skin is highly localized to the site of injection this was an attractive model in which to test the ability of antisense oligonucleotides to inhibit IL-1 receptor-mediated responses. Of great interest, therefore, was the observation that antisense oligodeoxynucleotides directed against IL-1 receptors blocked IL-1 stimulated neutrophil infiltration in vivo, one of a number of cellular responses in the complex cytokine-mediated inflammatory cascade in mouse dermal tissue. Injection of IL-1 into skin is associated within a few hours with infiltration of neutrophils into the site (20, 21) without edema (21). This effect of IL-1 is a consequence of the activation of type I IL-1 receptors on endothelial cells and subsequent increase in the number of adhesion molecules expressed by these cells (21, 36, 37). It is equally likely that activation of IL-1 receptors increases synthesis of chemotactic eicosanoids by connective tissue cells. These cells would be a primary target of the murine antisense oligonucleotides employed in our studies. It is unlikely that IL-1 receptors on neutrophils would have been affected as these cells are thought to exclusively express type II IL-1 receptors which have been recently demonstrated by cloning to share only ~ 28% amino acid homology to the type I receptor (38). In addition, it is unlikely that intradermal antisense oligonucleotide would have significant access to neutrophils in the blood. While future studies are underway to identify the cell types in mouse dermal tissue that were targets of antisense oligonucleotides to the IL-1 receptor, this is the first report of the successful application of these agents in vivo in a system with potential therapeutic application.

Many hurdles remain, however, before the therapeutic potential of antisense oligonucleotides can be reduced to practice. Present synthetic methodologies are expensive and limited data exist on the in vivo pharmacokinetics and bioavailability of these molecules (39). Speculating that the dose-response data presented in these studies can be translated to an adult human, and, assuming that the volume of distribution of an S-oligo is equal to plasma volume, then a single dose of 50 mg of hS-oligo-1 should be sufficient to reach an effective plasma concentration, thereby making antisense oligonucleotides comparable to many other classes of therapeutic agents. Of course, use of specialized delivery vehicles, such as targeted liposomes, may significantly reduce the required dose and potentially enhance biological effectiveness.

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