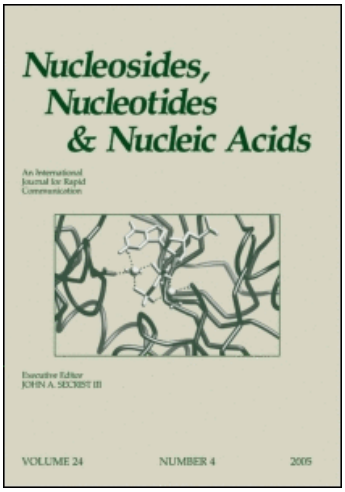

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Antisense Strategies for Inhibition of Tumor Necrosis Factor-α Synthesis

Gunther Hartmann[a]; Anne Krug[a]; Martin Bidlingmaier[a]; Andreas Eigler[a]; Stefan Endres[a]
[a] Medizinische Klinik, Klinikum Innenstadt of the Ludwig-Maximilians-University, y, Germany

To cite this Article Hartmann, Gunther , Krug, Anne , Bidlingmaier, Martin , Eigler, Andreas and Endres, Stefan(1997) 'Antisense Strategies for Inhibition of Tumor Necrosis Factor-α Synthesis', Nucleosides, Nucleotides and Nucleic Acids, 16: 5, 629 — 634
To link to this Article: DOI: 10.1080/07328319708002927
URL: http://dx.doi.org/10.1080/07328319708002927

NUCLEOSIDES & NUCLEOTIDES, 16(5&6), 629-634 (1997)

ANTISENSE STRATEGIES FOR INHIBITION OF TUMOR NECROSIS FACTOR-α SYNTHESIS

Gunther Hartmann, Anne Krug, Martin Bidlingmaier, Andreas Eigler, and Stefan Endres*
Medizinische Klinik, Klinikum Innenstadt of the Ludwig-Maximilians-University
Munich, Germany

ABSTRACT The proinflammatory cytokine tumor necrosis factor-α (TNF) plays a key role in inflammatory disease. Antisense oligonucleotide-mediated inhibition of monocyte-derived TNF synthesis may provide a valuable tool for therapeutic intervention. We established a model allowing specific suppression of TNF synthesis by oligonucleotides.

INTRODUCTION

The cytokines tumor necrosis factor-α (TNF), interleukin-1 and interleukin-6 are known to be potent mediators of inflammation. Recent studies demonstrated that TNF synthesis is tightly regulated in a hierarchical manner. In several studies, specific blockade of TNF function by antibodies revealed a necessary role for TNF in inflammatory disease such as rheumatoid arthritis, Crohn's disease and the Jarisch-Herxheimer reaction. However, repeated application of anti-TNF antibodies caused side-effects. Therefore the development of other strategies for TNF suppression gains importance. Current concepts for pharmacologic inhibition of TNF synthesis are reviewed by Eigler et al.[1]. In this report we describe our efforts to apply the antisense technique for specific suppression of TNF synthesis.

While the principle of the antisense mechanism has been unequivocally proven by expression of antisense transcripts and by injection of antisense oligonucleotides into the cytoplasm of single cells, there remain two major obstacles for the application of exogenous oligonucleotides: the stability of oligonucleotides against nucleases; the uptake of oligonucleotides into cells and penetration through cellular membranes. Stability is achieved by chemical modification of oligonucletides (i.e. phosphorothioate modification).

Modification, however, can reduce specifity of binding to the target RNA and induce non-specific effects. Cationic lipids (i.e. lipofectin) are known to effectively enhance cellular uptake of oligonucleotides. Complexes of oligonucleotides and cationic lipids bind to and penetrate cellular membranes. Oligonucleotides are subsequently released into the cytoplasm and the nuceus. Application of cationic lipids is limited by toxicity, and so far, systemic administration of cationic lipids has not been efficient.

Monocytes are target cells for anti-inflammatory antisense strategies currently tested in clinical trials. This cell type is characterized by strong phagocytic and catalytic activity which may limit the intracellular availability of antisense oligonucleotides. We studied interactions between monocytes and antisense oligonucleotides with respect to non-specific oligonucleotide effects, oligonucleotide incorporation and antisense-mediated inhibition of gene expression.

METHODS

Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy fasting volunteers by Ficoll-Hypaque gradient centrifugation. Cells were seeded at a final concentration of 2.5 x 10^6/ml in flat bottom 96-well microtiter plates (200 µl/well). TNF synthesis was stimulated by lipopoysaccharide (LPS) diluted to a final concentration of 10 ng/ml. TNF concentrations were determined by specific radioimmunoassay.

Phosphorothioate-modified oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). Mismatched oligonucleotides are used as controls (TABLE 1).

For fluorescence microscopy, cells were incubated with oligonucleotides conjugated to rhodamine at the 5´-end, were placed on a chambered coverglass (Nunc Inc., Naperville, USA) and analysed with a conventional fluorescence microscope (Diaphot TMD, Nikon) or a confocal laser microscope (Zeiss, Oberkochen, Germany). The cationic lipid lipofectin consists of equal parts of DOTMA (n-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride; monovalent cationic lipid) and DOPE (dioleoyl phosphotidylethanolamine; not charged).

RESULTS

Induction of LPS-stimulated tumor necrosis factor synthesis

It has been described that oligonucleotides can activate murine B-lymphocytes [2]. We examined the influence of oligonucleotides on LPS-stimulated tumor necrosis factor-α (TNF) synthesis in freshly isolated human peripheral blood mononuclear cells [3]. The addition of completely phosphorothioate-modified oligonucleotides (5 µM; 18mer) caused amplification of TNF synthesis up to 410 % compared to the control with LPS alone. This effect was dose-dependent. Without LPS stimulation, phosphorothioate oligonucleotides

TABLE 1. Characteristics of the oligonucleotides used

Name	Sequences	Mismatches
Anti-TNF-in	5` CAT GCT TTC AGT GCT CAT 3` / 0	0
Anti-TNF-in-2m	5` CAT GGT TTC AGT CCT CAT 3` / 2	2
Anti-TNF-in-4m	5` CAT GGT TTG ACT CCT CAT 3` / 4	4
Anti-TNF-in scrambled	5` TAC TGC AGG ATT CTC TTC 3`	

Anti-TNF translation initiation region oligonucleotide (*anti-TNF-in*) and its mismatch controls have the same content of nucleotides (G plus C content 45 %). Mismatched nucleotides are underlined. *Anti-TNF-in* is complementary to nucleotides 796 to 813 spanning the initiation region of TNF mRNA.

A, adenosine; G, guanosine; C, cytosine; T, thymidin

did not induce TNF production. We demonstrated that the enhancement of LPS-stimulated TNF production by phosphorothioate oligonucleotides does not rely on the intracellular presence of oligonucleotides and is not mediated by LPS contamination. Partially phosphorothioate-modified oligonucleotides (4 phosphorothioate-modified linkages) and unmodified oligonucleotides did not increase of TNF synthesis. Oligonucleotide-provoked enhancement of TNF synthesis is prevented in the presence of the cationic lipid lipofectin and reversed by high concentrations of the polyanion heparin. Our data suggest that amplification of TNF synthesis may be caused by binding of the polyanionic phosphorothioate oligonucleotide to cationic sites on the cell surface.

Oligonucleotide uptake in monocytes

We studied the incorporation and intracellular distribution of oligonucleotides in monocytes in order to establish an experimental model for effective antisense suppression of TNF. The cationic lipid lipofectin enhances oligonucleotide uptake by forming complexes with the polyanionic oligonucleotide molecules. We compared spontaneous uptake of oligonucleotides to lipofectin-mediated uptake. Monocytes were incubated with rhodamine-labeled oligonucleotides (1 μM) alone or together with lipofectin (25 μg/ml). Successive optical slices from the top to the bottom of cells were visualized by confocal laser microscopy. In the absence of lipofectin, fluorescence staining in cytoplasmatic vesicles of monocytes but no nuclear staining was observed. In the presence of lipofectin, oligonucleotides accumulated in the nuclei of monocytes.

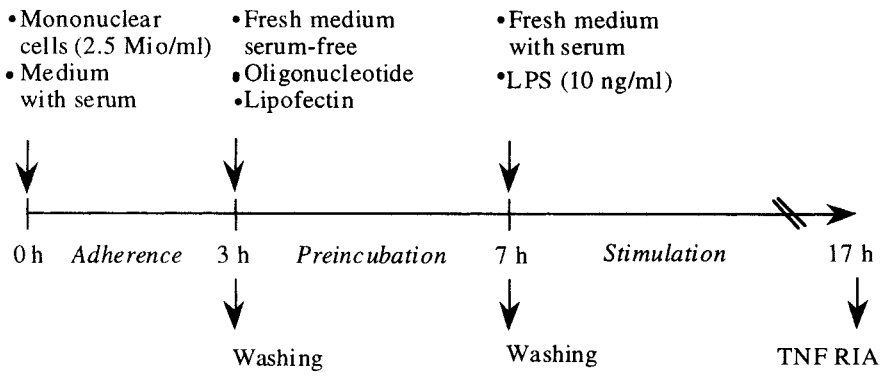

FIG. 1. Experimental protocol for lipofectin-facilitated suppression of LPS-induced TNF synthesis by antisense oligonucleotides in human monocytes.

Freshly prepared human peripheral blood mononuclear cells were allowed to adhere to the bottom of the cell culture plate (*adherence period* 3 h). Subsequently not-adherent cells were removed by washing and medium exchange. In the following preincubation period serum-free medium containing lipofectin (10 μg/ml) and oligonucleotide (125 nM to 2 μM) was added to the remaining cells. After 4 h cells were washed again, serum-containing medium was added and TNF synthesis was stimulated with 10 ng/ml LPS. After additional 10 h TNF was measured in combined cell lysate and supernatant by specific radioimmunoassay.

Antisense-mediated inhibition of tumor necrosis factor synthesis

The protocol allowing specific antisense-mediated TNF suppression *in vitro* is shown in FIG. 1.

Two parts of the protocol were found to be especially important: the *serum-free* preincubation with oligonucleotide and lipofectin and the separation of the preincubation from the stimulation with LPS by washing the cells. Anti-TNF translation initiation region oligonucleotide (*anti-TNF-in*) applied according to this protocol was the most effective sequence [4]. *Anti-TNF-in* is an 18-mer oligonucleotide complementary to nucleotides 796 to 813, spanning the translation initiation region of TNF mRNA. *Anti-TNF-in* contains no CpG-dinucleotide and not more than two neighboring C- or G-nucleotides, motifs which have been shown to confer a high rate of nonspecific oligonucleotide effects.

Applying this protocol we found specific and reproducible suppression of TNF synthesis by *anti-TNF-in* . This was demonstrated by the results of independent experiments with cells from different healthy volunteers (FIG. 2).

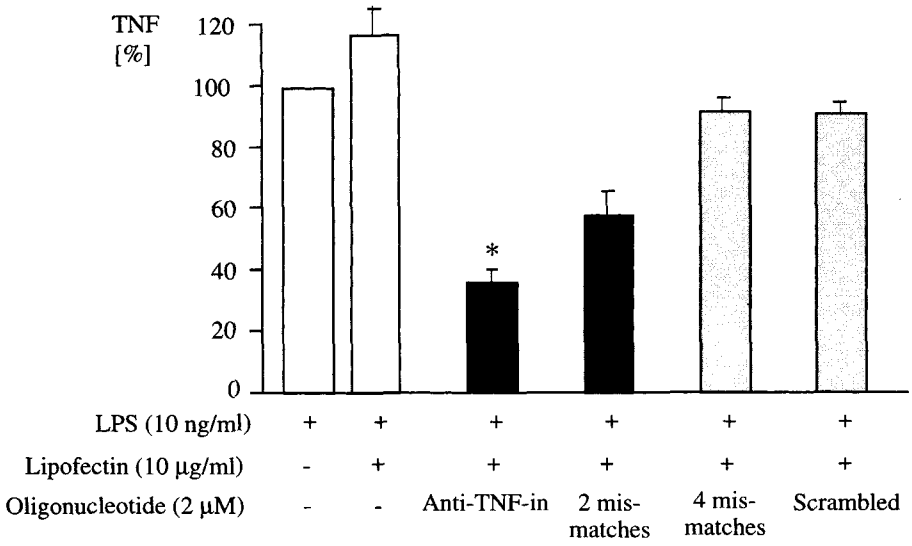

FIG. 2. Suppression of TNF synthesis by *anti-TNF-in* .
The results of six independent experiments using monocytes derived from different volunteers are demonstrated. The mean of TNF synthesis out of six independent experiments is set 100 % (left white bar). The control experiments were carried out in duplicates (figure from Hartmann et al; 1996b).

The mean of six experiments showed a suppression of LPS-stimulated TNF synthesis by 65 % ± 17 % (2 µM *anti-TNF-in*, p = 0.015). The control oligonucleotide with 2 mismatches (*anti-TNF-in-2m*) resulted in suppression of TNF synthesis by 42 % (2 µM). The control oligonucleotide with 4 mismatches (*anti-TNF-in-4m*) and the scrambled control did not lead to suppression of TNF synthesis. The control experiment with lipofectin alone revealed a small enhancement of LPS-stimulated TNF synthesis, which was not significant (15 %, p = 0.099).

In conclusion, our results of non-specific TNF induction by oligonucleotides bear relevance to all *in vitro* studies attempting to influence protein synthesis by antisense oligonucleotides. The significance of these findings for *in vivo* application of phosphorothioates in situations presenting a stimulus for TNF synthesis, such as sepsis, remains to be elucidated. Induction of TNF by oligonucleotides may hamper antisense-mediated TNF inhibition. Induction of TNF can be prevented using partially phosphorothioate-modified oligonucleotides or the cationic lipid lipofectin. In addition, lipofectin quantitatively facilitates oligonucleotide incorporation in monocytes and improves

nuclear availability of oligonucleotides. The presented results demonstrate that TNF inhibition by antisense oligonucleotides is feasible if the mentioned prerequisites are addressed. Our results form the basis for experimental settings aiming at antisense-mediated TNF inhibition *in vivo*.

This study was supported by grant No. 93.042.2 from the Wilhelm Sander-Stiftung

REFERENCES

1. Eigler A, Sinha B, Hartmann G, Endres S: Taming TNF: anti-tumor necrosis factor strategies. Immunol Today (in press) 1997

2. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM: CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374: 546, 1995

3. Hartmann G, Krug A, Waller-Fontaine K, Endres S: Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: Dependence on phosphorothioate modification and reversal by heparin. Mol Med 2: 429, 1996a

4. Hartmann G, Krug A, Eigler A, Moeller J, Murphy J, Albrecht R, Endres S: Specific suppression of human tumor necrosis factor-α synthesis by antisense oligodeoxynucleotides. Antisense Nucl Acid Drug Dev (in press) 1996b