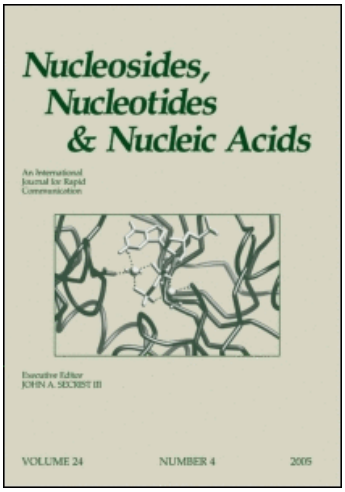

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Oligonucleotide uptake in leukocytes is dependent on extracellular calcium: a hint for the involvement of adhesion molecules?

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OLIGONUCLEOTIDE UPTAKE IN LEUKOCYTES IS DEPENDENT ON EXTRACELLULAR CALCIUM: A HINT FOR THE INVOLVEMENT OF ADHESION MOLECULES?

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ABSTRACT An enhanced appreciation of internalization of antisense oligonucleotides will extend possible applications in experimental and therapeutic settings. We found that oligonucleotide incorporation in monocytes, granulocytes and B-lymphocytes but not in T-lymphocytes is strongly dependent on the presence of extracellular calcium, and is inhibited by heparin. Our results support the hypothesis that calcium-dependent adhesion molecules mediate oligonucleotide internalization in leukocytes.

INTRODUCTION

Leukocytes are important target cells for both antisense and non-antisense strategies. Leukocytes are involved in antisense strategies directed against inflammation, viral infection and hematologic disorders[1-3]. Non-antisense strategies include the application of immunostimulatory effects of defined oligonucleotide sequences in different therapeutic settings[4, 5]. Furthermore, non-specific immunostimulatory side effects may complicate the application of therapeutic antisense oligonucleotides[1, 2, 6]. We examined the interaction between oligonucleotides and leukocytes with respect to immunostimulation and cellular uptake.

Both, oligonucleotides and polysaccharides (heparin, heparan sulfate), are polyanions and share structural simularities (**FIG. 1**). The cell surface of leukocytes is negatively charged. Adhesion molecules such as integrins mediate the contact to negatively charged

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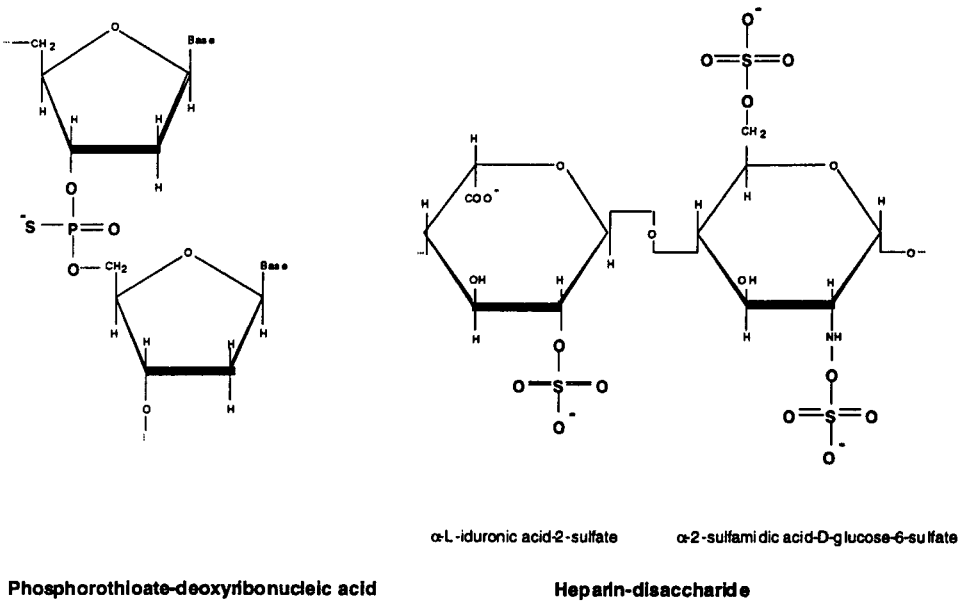

FIG. 1. Structural similarities between the polyanions phosphorothioate-deoxyribonucleic acid and heparin.

In phosphorothioate-modified oligodeoxynucleic acid one free oxygen atom of the phosphate is replaced by a sulfur atom providing increased stability against nucleases. "Base" indicates the linkage to one of the bases adenosine, guanosine, cytosine and thymidin. For heparin one of several possible sulfated disaccharides of the polysaccharide chain is shown. Both heparin and phosphorothioat-deoxyribonucleic acid represent polyanions. Negative charges are indicated with bold letters.

components of the extracellular matrix (heparan sulfate, keratan sulfate)[7]. Positively charged so-called heparin binding sites bind to polyanionic molecules. The function of adhesion molecules is dependent on the presence of extracellular calcium. The binding of leukocytes to extracellular matrix induces a co-stimulatory signal[7-9]. In contrast, heparin-binding does not induce co-stimulation[10]. Oligonucleotides, polyanionic molecules, may bind to similar sites at the cell surface than heparin or components of the extracellular matrix[11] **(FIG. 2)**.

It has been proposed that oligonucleotide uptake in leukocytes is mediated by binding to adhesion molecules and subsequent internalization. Recently it has been demonstrated for granulocytes, that oligonucleotides bind to the adhesion molecule MAC-1, and that this binding is dependent on extracellular calcium, furthermore, that oligonucleotide binding to

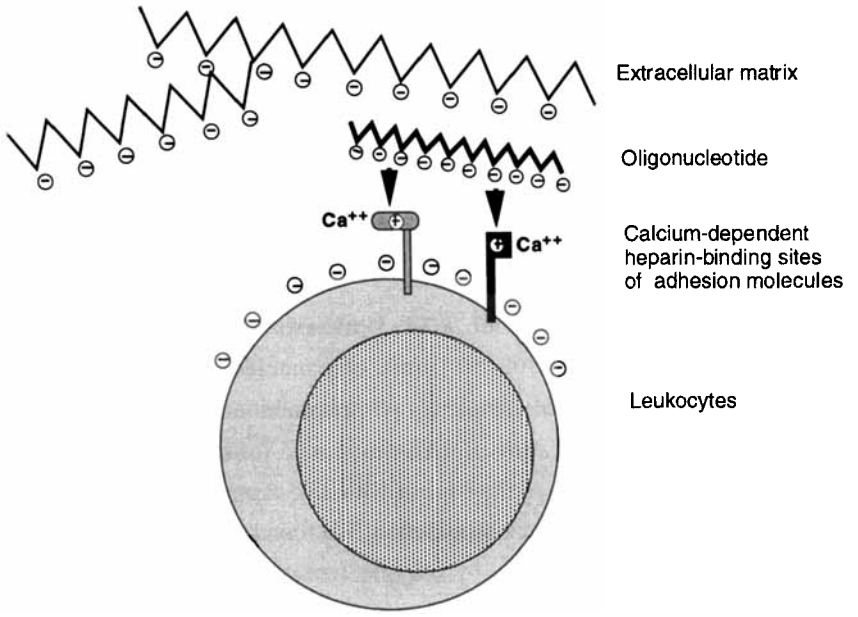

FIG. 2. Oligonucleotide bind to adhesion molecules of the cell surface.
The surface of leukocytes is negatively charged. Polyanions of the extracellular matrix (heparane sulfate, keratane sulfate) bind to positively charged binding sites of adhesion molecules (calcium-dependent). Binding of leukocytes to extracellular matrix induces a co-stimulatory signal. The polyanion heparin binds to the same sites without inducing a co-stimulatory signal. Oligonucleotides are also polyanions which may bind to similar sites potentially inducing a co-stimulatory signal.

MAC-1 is responsible for cellular uptake and immunostimulatory effects of oligonucleotides (induction of reactive oxygen species)[12, 13]. This points to a possible relation between oligonucleotide uptake and immunstimulation. We hypothesized that heparin-binding sites on adhesion molecules bind oligonucleotides in a calcium-dependent manner.

METHODS

For studying oligonucleotide uptake, human peripheral blood mononuclear cells (PBMC) or samples of whole blood were incubated for 3 h with a fluorescein-labeled phosphorothioate oligonucleotides (5` TAC TGC AGG ATT CTC TTC 3`), EDTA and heparin as indicated. After extensive washing incorporation of oligonucleotides in

leukocyte subpopulations was quantified by flow cytometry. Subpopulations of leukocytes were identified by morphologic characteristics (granulocytes) or by staining with phycoerythrin-labeled anti-CD-antibodies (CD14 for monocytes, CD3 for T-lymphocytes and CD19 for B-lymphocytes). In our system the lowest EDTA concentration providing complete anticoagulation of whole blood was 0.8 mM.

RESULTS

Heparin reverses enhancement of TNF synthesis by oligonucleotides.

Previously we found that phosphorothioate oligonucleotides co-stimulate human mononuclear cells. The addition of completely phosphorothioate-modified oligonucleotides (5 μM; 18mer) caused amplification of LPS-stimulated tumor necrosis factor-α (TNF) synthesis up to 410 % compared to the control with LPS alone [6]. We further investigated the mechanism responsible for this co-stimulation. We found that increasing concentrations of heparin reverse the co-stimulation by oligonucleotides (**FIG. 3**). Therefore, heparin sharing similar structural characteristics may replace oligonucleotides from their binding sites at the cell surface. To test this we examined oligonucleotide uptake in leukocytes.

Oligonucleotide uptake in leukocyte subpopulations.

We examined oligonucleotide uptake in both isolated mononuclear cells and in whole blood. The amount of oligonucleotides associated to monocytes was 2-fold higher than in granulocytes, and 5-fold higher than in B-lymphocytes. T-lymphocytes did not incorporate oligonucleotides (close to autofluorescence). With respect to our results of inhibition of co-stimulation by heparin we examined the effect of heparin on oligonucleotide uptake.

Heparin inhibits oligonucleotide uptake in leukocytes.

The addition of heparin inhibited oligonucleotide uptake in monocytes, granulocytes and B-lymphocytes in a concentration dependent manner up to 16 IU/ml. Heparin concentrations higher than 16 IU/ml did not further inhibit oligonucleotide uptake. Inhibition of oligonucleotide uptake by heparin point to an involvement of heparin-binding sites at cell surface proteins. Adhesion molecules are surface proteins with heparin-binding sites. The function of adhesion molecules is dependent on the presence of extracellular calcium. We evaluated the role of extracellular calcium on oligonucleotide uptake.

Oligonucleotide uptake in leukocytes is dependent on extracellular calcium.

The anticoagulant EDTA strongly binds 2-fold positive cations in an equimolar manner. The addition of 4 mM calcium completely binds extracellular calcium (Ca^{++} 2.1 to 2.6

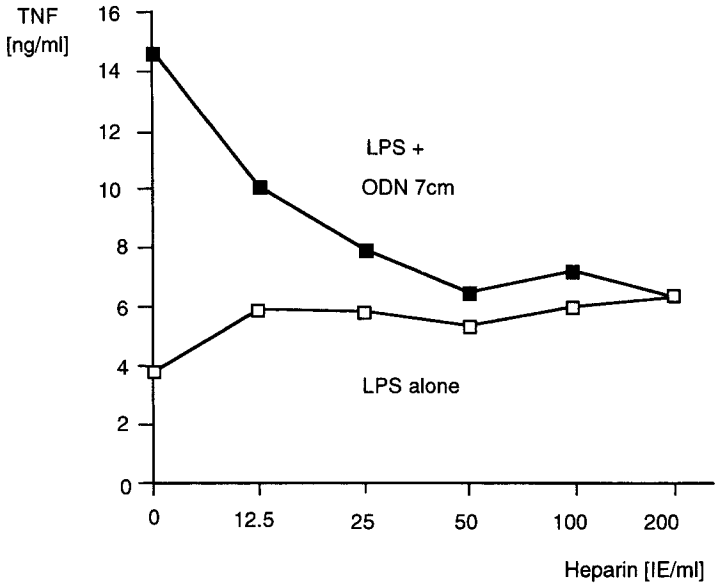

FIG. 3. Heparin reverses enhancement of TNF synthesis by oligonucleotides. Human mononuclear cells (2.5 Mio/ml) were stimulated with 10 ng/ml LPS for 20 h. The influence of increasing concentrations of heparin on the enhancement of tumor necrosis factor (TNF) production by phosphorothioate oligonucleotides (5 μM) was tested. At a concentration above 25 IE/ml heparin reverses enhanced TNF synthesis to the level of the control with LPS alone. Reversal by heparin suggests displacement of oligonucleotides from the binding sites that mediate enhanced TNF synthesis. Influence of heparin on TNF measurement by the RIA was excluded (Fig. adapted from [6].

mM, combined free and protein bound) in plasma. The minimal EDTA concentration required for complete anticoagulation of whole blood was 0.8 mM. The addition of higher EDTA concentrations decreased oligonucleotide incorporation in monocytes (**FIG. 4**) and in granulocytes and B-lymphocytes (data not shown). In preliminary experiments, the addition of increasing concentrations of extracellular calcium reversed uptake inhibition by EDTA.

In conclusion, our results argue for the involvement of calcium-dependent heparin-binding sites in oligonucleotide uptake of leukocytes. Such sites are located at adhesion molecules such as integrins. Beside oligonucleotide incorporation, binding to these sites is necessary for co-stimulatory action of phosphorothioate oligonucleotides, but it is yet unknown whether binding to these sites is sufficient. Two major questions remain to be

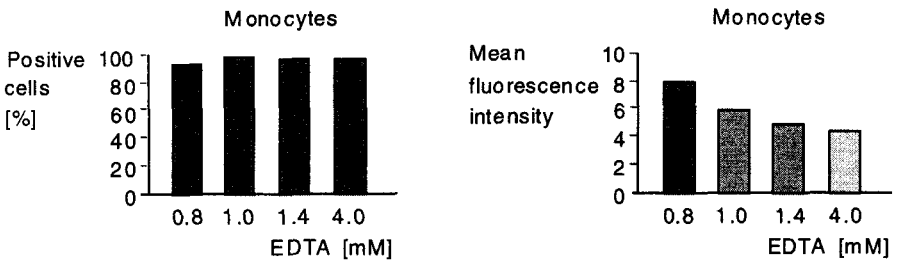

FIG. 4. EDTA inhibits oligonucleotide uptake in monocytes.
Whole blood samples were incubated with fluorescein-labeled oligonucleotide (500 nM) in the presence of increasing concentrations of EDTA. Oligonucleotide incorporation is represented by the percentage of oligonucleotide positive cells (left panel) and mean fluorescence intensity per cell (right panel). Results are shown as means of experimental duplicates.

answered: a) which adhesion molecules are involved in oligonucleotide binding, and b) is the co-stimulatory action of oligonucleotides directly mediated by binding to adhesion molecules, or does binding to adhesion molecules and the subsequent internalization enhance availability of oligonucleotide to an intracellular protein responsible for co-stimulation?

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