DNA Apatmers Block L-Selectin Function In Vivo
Inhibition of Human Lymphocyte Trafficking in SCID Mice

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Abstract

Selectins participate in the initial events leading to leukocyte extravasation from the blood into tissues. Thus the selectins have generated much interest as targets for antiinflammatory agents. Therapeutic molecules based on the monomeric carbohydrate ligand sialyl Lewis X (SLea) have low affinities and are not specific for a given selectin. Using SELEX (Systematic Evolution of Ligands by EXPonential Enrichment) technology, we have generated aptamers specific for L-selectin that require divalent cations for binding and have low nanomolar affinity. In vitro, the deoxyoligonucleotides inhibit L-selectin binding to immobilized SLea in static assays and inhibit L-selectin–mediated rolling of human lymphocytes and neutrophils on cytokine-activated endothelial cells in flow-based assays. These aptamers also block L-selectin–dependent lymphocyte trafficking in vivo, indicating their potential utility as therapeutics. (J. Clin. Invest. 1996. 98:2688–2692.) Key words: oligonucleotide * inflammation • cell adhesion • therapeutics • SELEX

Methods

SELEX. An L-selectin–IgG fusion protein (LS-Rg) was produced and SELEX was performed as described (23 and references therein), with the following modifications. All selections were performed at 22°C except round 1 (4°C) and rounds 8, 13, 16, and 17 (37°C). Selected single-stranded DNAs (ssDNAs) were precipitated, PCR-amplified using a biotinylated 3′ PCR primer, and the strands were separated using denaturing polyacrylamide gel electrophoresis. The starting ssDNA pool contained a 40-nucleotide randomized region flanked by fixed sequences for primer annealing. 3′-taacetatagatc-tactaC-tactagatc-tactag-3′. Individual ligands were cloned and sequenced by standard procedures.

DNA-protein equilibrium dissociation constants. Kd were measured by nitrocellulose filter partitioning (20) in binding buffer (20 mM Hepes, pH 7.5, 125 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM KCl) plus 0.01% (wt/vol) human serum albumin (Sigma Chemical Co., St. Louis, MO) and were calculated by least square fits using Kaleidagraph (Synergy Software, Reading, PA).

Oligonucleotide synthesis and modification. DNAs were synthesized by Operon Technologies, Inc. (Alameda, CA) or at NeXstar Pharmaceuticals using standard procedures. Fluorescein labeling was accomplished by incorporation of an FITC phosphoramidite (Glen

1. Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cells; LS-Rg, L-selectin-Rg; MLN, mesenteric lymph nodes; PEG, polyethylene glycol; PLN, peripheral lymph nodes; SCID, severe combined immunodeficiency; SELEX, Systematic Evolution of Ligands by EXPonential Enrichment; SLea, sialyl Lewis X; ssDNA, single-stranded DNA.
Three DNAs

Table I. High Affinity, Specific Binding of Deoxyoligonucleotide Ligands of L-Selectin: Sequences, Affinity, and Specificity of

Lymphocyte trafficking. Human PMBC were purified from heparinized blood by a Ficoll-Hypaque gradient, washed twice with HBSS (calcium/magnesium free) and labeled with $^{51}$Cr (Amersham, Arlington Heights, IL). Cells were then washed twice with HBSS (containing calcium and magnesium) and 1% BSA (Sigma Chemical Co.). Labeled cells (2 x 10$^6$) were either untreated or mixed with either 15 pmol of antibody (DREG-56 or MEL-14) or 4 or 0.4 nmol of PE-coupled and 3′ capped aptamer, before intravenous injection into female SCID mice (6–12 wk of age). Alternatively, the mice were injected with either 15 pmol DREG-56 or 4 nmol modified oligonucleotide 1–5 min before injecting the labeled cells. 1 h later the animals were anesthetized, a blood sample was taken, and the mice were killed. Peripherally lymph nodes (PLN), mesenteric lymph nodes (MLN), Peyer’s patches, spleen, liver, lungs, thymus, kidneys, and bone marrow were removed and the counts incorporated into the organs determined by a gamma counter (Packard Instruments, Meriden, CT).

Results and Discussion

Selection of aptamers from a combinatorial DNA library. The binding specificity of selectins for their ligands appears to be mediated predominantly by the amino-terminal calcium-dependent lectin domain (25–27). This calcium dependence provides a method for preferentially isolating inhibitory aptamers, since oligonucleotides bound at or near the carbohydrate binding site can be selectively eluted with low concentrations of EDTA (23). The 2′ amino-pyrimidine oligonucleotides previously isolated by this procedure have high affinity at 4 and 22°C and much lower affinity at 37°C (23) and are hence unsuitable for in vivo testing. To isolate antagonists with improved thermal stability, we performed a SELEX experiment using deoxyoligonucleotides and higher selection temperatures, either 22 or 37°C. A starting pool of 10$^{15}$ random sequence ssDNAs was incubated with human LS-Rg immobilized on protein A–Sepharose beads. After extensive washing, bound oligonucleotides were eluted with 5 mM EDTA and PCR-amplified. DNA strands were then separated and the cycle was repeated. After 15 iterations, the DNA pool bound to LS-Rg with a $K_a$ of 0.9±0.1 nM, versus > 5 μM for the starting random pool (data not shown).

Sequence, affinity, and specificity of aptamers. Cloning and sequencing of aptamers from the 15th and 17th rounds revealed three distinct sequence families (Hicke, B., and D. Parma, manuscript in preparation). Individual ligands had high affinity and specificity for LS-Rg; binding was divalent cation-dependent (data not shown). A representative aptamer from each sequence family is shown in Table I. Ligand LD201 bound to LS-Rg with a $K_a$ of 1.8 nM at 37°C. The affinities of

Table I. High Affinity, Specific Binding of Deoxyoligonucleotide Ligands of L-Selectin: Sequences, Affinity, and Specificity of Three DNAs

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sequence</th>
<th>$K_d$ (nM)</th>
<th>$K_a$/$K_d$</th>
<th>$K_p$/$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD201</td>
<td>5′-CAAGGTAACCAGTACAAAGTGGTCTAACGTATGGCTTC3′</td>
<td>1.8±0.2</td>
<td>300</td>
<td>9000</td>
</tr>
<tr>
<td>LD174</td>
<td>5′-CATTCCACATGCGCCCTCTTCATGCATGTTCGCGGT3′</td>
<td>5.5±5.1</td>
<td>600</td>
<td>8000</td>
</tr>
<tr>
<td>LD196</td>
<td>5′-TGGCGGTCAGGGCCGCGTACACCATCTACCTGGGAAGTGA3′</td>
<td>3.1±0.4</td>
<td>200</td>
<td>15000</td>
</tr>
</tbody>
</table>

Sequences, representative of the three sequence families, do not include the 5′ and 3′ fixed sequences (see Methods). Specificity is the ratio of $K_a$ for E-selectin–Rg (E) to L-selectin–Rg (L) and for P-selectin–Rg (P) to L, as measured by a nitrocellulose filter binding assay. Values shown represent the mean±SE from ≥ 2 experiments.
LD174 and LD196 were comparable. No binding was detected to 700 nM concentrations of either wheat germ agglutinin, an N-acetyl glucosamine/sialic acid binding plant lectin, or CD22-IgG chimera, a mammalian sialic acid binding lectin (28). These ligands bound to LS-Rg 200–600-fold more tightly than to human E-selectin–Rg and 8,000–15,000-fold more tightly than to human P-selectin–Rg (Table I). In contrast to SLe\(^x\), the aptamers described here are specific for L-selectin and bind with low nanomolar affinity.

**Inhibition of SLe\(^x\) binding and competition with a blocking mAb.** We next determined that the DNAs inhibited L-selectin binding to SLe\(^x\). Shortened forms of LD201, LD174, and LD196 were prepared (see Methods for sequences). These truncated forms, LD201t1, LD174t1, and LD196t1 (data not shown) inhibited LS-Rg binding to immobilized SLe\(^x\) with IC\(_{50}\)s of \(\sim 3\) nM (Fig. 1A). This is a \(10^5–10^6\)-fold improvement over the published IC\(_{50}\) values for SLe\(^x\) in similar plate-binding assays (9–11, 23). A scrambled sequence based on LD201t1 showed no activity in this assay. Binding of all three DNA ligands to LS-Rg was blocked by DREG-56, an L-selectin blocking monoclonal antibody (29), but not by an isotype-matched control (Fig. 1B). In competition experiments, LD201t1, LD174t1, or LD196t1 prevented radiolabeled LD201t1 from binding to LS-Rg, consistent with the premise that the ligands bind the same or overlapping sites (data not shown). The blocking and competition experiments, taken together with divalent cation dependence of binding, suggest that all three aptamers bind to the lectin domain. This conclusion has been verified for LD201 by cross-linking experiments (Hicke, B., and D. Parma, manuscript in preparation).

**Aptamer binding to cell surface L-selectin.** FITC-conjugated LD201t1 specifically bound human lymphocytes and neutrophils (Fig. 2A) and granulocytes (Fig. 2B) in whole blood. Cells were stained with FITC-LD201t1 alone and in the presence of 0.3 \(\mu\)M DREG-56, 7 \(\mu\)M unlabeled LD201t1, or cells were reasayed after addition of 4 mM EDTA. Cells were gated using side scatter and CD45-Cy5PE staining.

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**Figure 1.** The oligonucleotides block L-selectin’s SLe\(^x\) binding site. (A) Inhibition of LS-Rg binding to SLe\(^x\). Immobilized SLe\(^x\) was incubated with LS-Rg and increasing concentrations of DNAs, along with a peroxidase-conjugated anti–human IgG. After washing, bound LS-Rg was indirectly quantified by addition of a peroxidase substrate and detection at 450 nM. Values shown represent the mean ± SE from duplicate, or triplicate, samples from one representative experiment. (B) Inhibition of oligonucleotide binding to LS-Rg by the adhesion blocking mAb DREG-56. 5\(^3\)P-labeled oligonucleotides (5 nM) were incubated with 1 nM LS-Rg and increasing concentrations of DREG-56 or an isotype-matched control. Reaction mixtures were incubated at 37°C for 15 min, partitioned by nitrocellulose filtration, and bound oligonucleotide was quantified. Open squares, LD201t1 plus control antibody; filled squares, LD201t1 plus DREG-56; open triangles, LD174t1 plus control antibody; closed triangles, LD174t1 plus DREG-56; open circles, LD196t1 plus control; filled circles, LD196t1 plus DREG-56. The data shown are representative of two experiments.

**Figure 2.** LD201t1 binds specifically to L-selectin on human lymphocytes (A) and granulocytes (B) in whole blood. Cells were stained with FITC-LD201t1 alone and in the presence of 0.3 \(\mu\)M DREG-56, 7 \(\mu\)M unlabeled LD201t1, or cells were reasayed after addition of 4 mM EDTA. Cells were gated using side scatter and CD45-Cy5PE staining.
phils in whole blood (Fig. 2); binding was inhibited by competition with DREG-56, unlabeled LD201, and by the addition of 4 mM EDTA (Fig. 2). In addition, human PBMC bound radiolabeled LD201. The binding was saturable, divalent cation-dependent, and competed by DREG-56 but not by an isotype-matched control antibody (data not shown). These cell binding studies demonstrated that the aptamers bound saturably and specifically to human L-selectin in the context of lymphocyte and neutrophil cell surfaces.

**Inhibition of lymphocyte rolling on activated endothelial cells.** To effectively block L-selectin-mediated adhesion in vivo, an antagonist must function in systems that are subject to hydrodynamic shear (5, 24). Accordingly, LD201t1 and the scrambled sequence, modified by addition of a 3'-capping group and a 5' 20,000 mol wt PEG (neither modification significantly alters aptamer affinity; data not shown), were studied in a flow system in vitro. In this system human PBMC “roll” on activated endothelial cells (HUVEC; activated with IL-1β) and rolling is dependent on both L-selectin and E-selectin (24). LD201t1 and DREG-56 blocked rolling to a similar extent, 70% for PBMC (Fig. 3 A) and ~50% for neutrophils (data not shown). The scrambled sequence had no activity in this assay (Fig. 3 A).

**Aptamer activity in vivo: lymphocyte trafficking in SCID mice.** As the aptamers bound to human but not rodent L-selectin (data not shown), a xenogeneic lymphocyte trafficking system was established to evaluate in vivo efficacy. Human PBMC, labeled with 51Cr, were injected intravenously into SCID mice. In this system, human cells traffic to PLN and MLN. Lymphocyte accumulation in MLN and PLN is inhibited by DREG-56 (Fig. 3 B) but not by MEL-14 (data not shown), a monoclonal antibody that blocks murine L-selectin–dependent trafficking (30). Cell trafficking was determined 1 h after injection. For trafficking experiments, 3'-capped and 5'-PEGylated ssDNA aptamers were used because pharmacokinetic studies in rats indicate that their half-life in plasma is ~18 min, significantly longer than that of unmodified ssDNA aptamers (Gill, S.C., personal communication).

In initial trafficking experiments, cells were incubated with either DREG-56 or 3'-capped and 5'-PEGylated oligonucleotide before injection. LD201t1 inhibited trafficking of cells to PLN (Fig. 3 B) and MLN (data not shown) in a dose-dependent fashion but had no effect on the accumulation of cells in other organs (data not shown). At the highest dose tested (4 nmol), inhibition by the oligonucleotide was comparable with the effect of DREG-56 (15 pmol) in this system. LD174t1 had similar activity (data not shown), while the scrambled sequence had no significant effect (Fig. 3 B). We next assayed the effect of modified oligonucleotide when it was not preincubated with cells. DREG-56 (15 pmol/mouse) or the modified oligonucleotide (4 nmol/mouse) was injected intravenously into animals and 1–5 min later the radiolabeled human cells
were given intravenously. Again, both LD201t1 and DREG-56 inhibited trafficking to PLN and MLN while the scrambled sequence had no effect (Fig. 3 C). Therefore, the modified oligonucleotide did not require preincubation with the cells to effectively block trafficking. To our knowledge, these experiments are the first demonstration of in vivo efficacy of an aptamer directed against a cell surface receptor.

In summary, we have generated oligonucleotide antagonists that bind with high affinity and specificity, in a divalent cation-dependent fashion, to human L-selectin. In vitro, the aptamers block binding of soluble L-selectin to SLe\(^\text{a}\), bind specifically to L-selectin on human leukocytes, and have 10\(^{-6}\)–10\(^{-5}\)-fold better affinity for L-selectin than does SLe\(^\text{a}\). In vivo, the aptamers block the trafficking of human lymphocytes to murine peripheral lymphoid tissues, making them superior to the previously described aptamer antagonists of L-selectin, the temperature sensitivity of which rendered them unsuitable for use in vivo (23). We expect to improve aptamer performance in vivo by enhancing affinity for L-selectin (Ringquist, S., manuscript in preparation) or by increasing resistance to nuclelease degradation, thereby providing high affinity, specific reagents that may block the interaction of L-selectin with its ligands in pathological states.

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References