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RNA and DNA aptamers as potential tools to prevent cell adhesion in disease

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Abstract

Recent research has shown that receptor-ligand interactions between surfaces of communicating cells are necessary prerequisites for cell proliferation, cell differentiation and immune defense. Cell-adhesion events have also been proposed for pathological conditions such as cancer growth, metastasis, and host-cell invasion by parasites such as *Trypanosoma cruzi*. RNA and DNA aptamers (aptus = Latin, fit) that have been selected from combinatorial nucleic acid libraries are capable of binding to cell-adhesion receptors leading to a halt in cellular processes induced by outside signals as a consequence of blockage of receptor-ligand interactions. We outline here a novel approach using RNA aptamers that bind to *T. cruzi* receptors and interrupt host-cell invasion in analogy to existing procedures of blocking selectin adhesion and function *in vitro* and *in vivo*.

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The SELEX technique

The SELEX method (systematic evolution of ligands by exponential enrichment) (1,2) is an oligonucleotide-based combinatorial library approach that has been extensively used to isolate high-affinity ligands (called aptamers) for a wide variety of proteins and small molecules (3-7). The *in vitro* selection of RNA and DNA ligands against specific targets obeys the same rules as natural selection. For this purpose a partial randomized synthetic DNA template is constructed containing a random inner region that is flanked on both sides by constant sequences. The random sequence classically consists of 15 to 75 random positions where all four bases are incorporated with equal probabilities. This pool containing 10^{12} to 10^{15} different sequences can be either directly used for selection or first transcribed to RNA using T7 RNA polymerase. In this case a T7 promoter site needs to be placed on the 5' site of the DNA template. The random DNA/RNA pool is exposed to the protein target and the best fitting molecules in the selection pool are culled and amplified. The procedure will be repeated with increasing stringency until the previous random pool is purified to a few molecules with the desired binding properties. The final pool is cloned into a bacterial vector and individual colonies are sequenced. The previous random regions are aligned and searched for consensus motifs. Consensus motifs often located in stem-loop structures are thought to mediate binding specificity. These aptamers are suitable for applications based on molecular recognition of a target molecule including diagnostics and therapeutics. Recently, the

use of the SELEX method has been extended to complex targets such as red blood cell membranes (8) and the membrane-bound nicotinic acetylcholine receptor (9), whole virus particles (10) and African trypanosomes (11). These ligands have dissociation constants in the picomolar to low nanomolar range for their protein targets. The SELEX methodology has also successfully been used to develop RNA antagonists of CD4 epitopes on mononuclear mouse cells (12) and selectin cell surface molecules (6,13). These selected RNA antagonists prevented the P-selectin-dependent neutrophil-platelet adhesion *in vitro*. In summary, today RNA or DNA aptamers can be obtained for almost every target whether complex or small.

The potential utility of aptamers as therapeutic agents is considerably enhanced by chemical modifications that lend resistance to nuclease attack (12,14). This resistance is either achieved by the addition of phosphorothioates (15) or by the substitution of the 2'-OH groups of pyrimidines with 2'-F, 2'-NH_2, or 2'-OMe. Aptamers recognize epitopes with the same specificity as antibodies but in contrast to antibodies they possess low immunogenicity and are not subject to proteolytic degradation. They can be easily modified at precise positions and reporter groups can be attached at the researcher's will.

Two promising approaches to synthesizing DNA/RNA aptamers directed against cell-adhesion molecules will be reviewed: RNA and DNA aptamers directed against L- and P-selectins and the development of RNA aptamers that interfere with the binding of host-cell matrix molecules to their receptors on *Trypanosoma cruzi* and affect cell invasion by *T. cruzi*, an ongoing research project of the authors.

RNA/DNA aptamers binding to selectins

P-, L-, and E-selectins form a homologous family of cell-adhesion molecules containing an amino terminal C-type lectin domain, an epidermal growth factor-like domain, two to nine complement-like repeats, a transmembrane domain, and a short cytoplasmic tail (16). Selectins mediate cell adhesion through calcium binding of the sialylated, fucosylated cell surface glycans that possess a sialyl-Lewis X-type structure and are involved in many cell-cell interactions in the vascular system. These molecules became targets of pharmacological and therapeutic approaches, since they participate in early steps of tissue damage in animal models of ischemia-reperfusion or inflammation (17). The best characterized ligand for P-selectin is P-selectin glycoprotein ligand-1. Since P-selectin antagonists were shown to reduce tissue damage in animal models resulting from stroke, reactions against heart transplantations, and atherosclerosis, an effort was made to evolve RNA aptamers that bind to P-selectins on cell surfaces and prevent the binding of natural ligands on communicating cells resulting in inhibition of cell adhesion (13). RNA and DNA aptamers have also been evolved against L-selectins binding with affinities to their targets in the picomolar to nanomolar range (6,18). The selected aptamers were tested *in vitro* as to their ability to inhibit selectin-mediated rolling of lymphocytes and neutrophils on cytokine-activated endothelial cells in flow-based assays. The cross-reactivity of aptamers directed against L- or P-selectins against other selectins was small. Both the aptamers directed against P- and L-selectins were 10^5 to 10^6 more effective in inhibiting adhesion of cell surface selectins to their natural ligands than sialyl-Lewis, a well-recognized natural oligosaccharide inhibitor of selectins (6,13). The high affinity and specificity of these aptamers for their targets may increase their future importance, since none of the known natural ligands of selectins is able to discriminate between the three selectin isoforms. *In vivo* experiments on mice with severe

combined immunodeficiency in which L-selectin function was blocked by DNA aptamers (18) resulted in inhibition of L-selectin-induced lymphocyte trafficking. The discussed studies reveal that RNA or DNA aptamers directed against cell-adhesion molecules are able to interrupt adhesion and cell communication *in vitro* and *in vivo*.

Selecting RNA aptamers that bind to cell-adhesion receptors on *T. cruzi*

Trypanosoma cruzi causing Chagas' disease is a protozoan that must invade host cells to complete its biological cycle. The parasite exists in three developmental forms: epimastigotes, that multiply extracellularly in the gut of insect vectors; amastigotes, the replicating intracellular mammalian stage of the parasite life cycle; an intermediate intracellular epimastigote-like form (19), and trypomastigotes as an extracellular form that disseminates and invades cells and transmits the infection from insects to mammals (20).

To contact and invade a mammalian host cell, trypomastigotes must cross a network of extracellular matrix proteins on the surface of cells of the blood-vessel walls and target organs. Following cell invasion, trypomastigotes transform into amastigotes and divide. Amastigotes transform back to trypomastigotes and, upon exiting the cell, infect new cells (19). Investigators accumulated evidence for receptor-ligand interactions in the early events of cell infection by trypanosomes (21). Host-cell matrix molecules (22-26) bind to the parasite surface and affect host-cell invasion.

Trypanosoma cruzi cell surface molecules have been isolated and characterized in our laboratory. These investigations resulted in the cloning and characterization of an 85-kDa protein that is specific for the trypomastigote stage (25-29). This glycoprotein, belonging to a family of related molecules denominated Tc-85, binds to the cell matrix protein laminin in a specific and saturable way (25,26). Tc-85 has extended sequence homology with the gp85 glycoprotein family that is related to the sialidase-trans-sialidase supergene family (30-32), suggesting that several members of the gp85 superfamily may have the ability to adhere to host-cell surface molecules. Monoclonal antibodies recognizing the Tc-85 glycoprotein of the trypomastigote surface prevented *Trypanosoma* invasion in cultured mammalian cells (28,33). Various investigations have shown the implication of proteins with molecular weights of about 85-90 kDa in the interaction of the parasites with the host cells (33-37).

Based on these observations, we propose the development of RNA aptamers that bind to the receptors of the cell matrix molecules laminin, thrombospondin, heparan sulfate and fibronectin on *T. cruzi* cell surfaces. As discussed for selectins, one would expect that the aptamers will only recognize the protein epitopes for which they have been selected, i.e., the natural receptors of laminin, fibronectin, thrombospondin, and heparan sulfate on the parasite surface.

A scheme for a proposed selection protocol is presented in Figure 1. A partially randomized DNA pool is transcribed *in vitro* in the presence of 2'-OH purines and 2'-F pyrimidines in order to create the initial pool of 10^{13} different nuclease-resistant RNA molecules. The RNA molecules are heated up and then slowly cooled down to room temperature to allow for proper folding. The RNA molecules are incubated with live trypomastigotes at a 100:1 RNA:binding site ratio. Unbound and weakly bound RNA molecules are washed off from the trypomastigotes and specifically bound RNA molecules are displaced using a cocktail of cell matrix molecules. The eluted RNA molecules are reverse transcribed to cDNA and amplified by PCR. The next generation SELEX pools contain an enriched fraction of RNA molecules that compete with host-cell matrix molecules for their binding sites

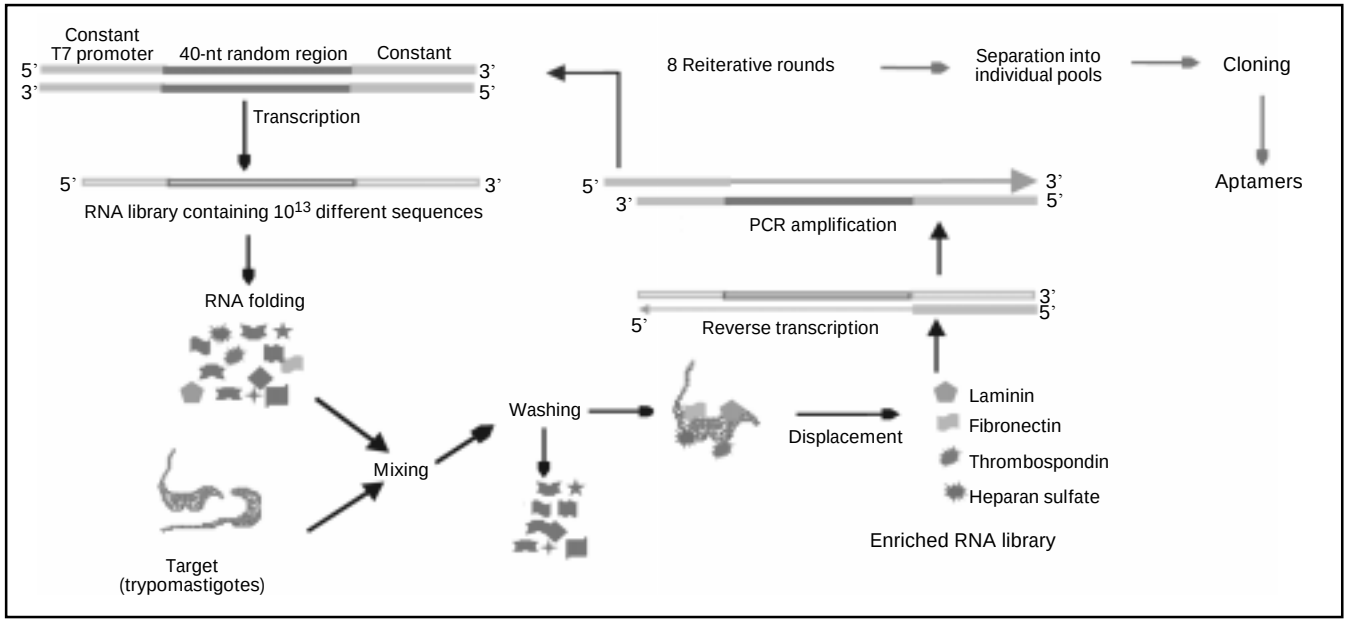

Figure 1 - Schematic representation of the systematic evolution of ligands by exponential enrichment (SELEX) protocol used for the isolation of RNA aptamers that bind to cell-adhesion receptors on *Trypanosoma cruzi*.

on *T. cruzi*. The procedure is repeated until no further purification can be achieved. At this stage, RNA molecules are constitutively eluted using individual cell matrix molecules as displacement agents. Individual aptamer sequences are cloned and searched for consensus motifs. One would expect that aptamers competing with a certain cell matrix molecule for receptor binding should share a common sequence motif. Determining the minimal RNA sequence that is necessary for receptor binding will help to find the most effective molecule.

Aptamers as tools to identify proteins involved in cell adhesion and invasion by parasites

The identification of receptors and ligands on parasite and host-cell surfaces may be important not only for elucidating invasion mechanisms used by *T. cruzi* but also for understanding the entry process of other pathogens that depend on adhesion to host-cell membranes.

Photocrosslinking of radiolabeled RNA ligands to their targets on parasite membranes can be used for ligand-mediated target purification (8). Using this method, any protein target on the parasite membrane, for which a high-affinity RNA ligand has been identified, can be purified. Microsequencing of the cross-linked RNA/receptor complexes will allow further isolation of cell surface proteins of the gp85 superfamily in *T. cruzi* and possibly also other parasite surface proteins involved in the invasion of host cells by these parasites.

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