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Selection of DNA aptamers against DC-SIGN protein

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Abstract Dendritic cells (DCs) are professional antigen-presenting cells and have come to be appreciated as critical controllers of the immune response, especially T cell responses. Apart from presenting antigens to T cells, DCs carry out many other functions in regulating immunity. DC-specific intercellular adhesion molecule (ICAM)-3 grabbing non-integrin (DC-SIGN) is a novel receptor that plays an important role in DC migration and adhesion, the inflammatory response, T cell activation, initiating the immune response, and immune escape of pathogens and tumors. DC-SIGN mediates DC binding to ICAM-3 on the T cell surface and ICAM-2 on the endothelial cell (EC) surface, and takes part in the initial interaction between DC and T cells or vascular ECs. The procedure of systematic evolution of ligands by exponential enrichment (SELEX) is a method in which single-stranded oligonucleotides are selected from a wide variety of sequences, based on their interaction with a target molecule. In this study, we selected DNA aptamers against DC-SIGN protein by SELEX, and measured their binding affinity for DC-SIGN. Finally, an appropriate aptamer with high affinity for DC-SIGN was obtained, and it blocked DC adhesion to ECs as effectively as anti-DC-SIGN monoclonal antibody.

Keywords Dendritic cells · DC-specific ICAM-3 grabbing non-integrin · Aptamer · SELEX

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Introduction

Aptamers are oligonucleotides (DNA or RNA) that can bind with high affinity and specificity to a wide range of target molecules, such as drugs, proteins, and other organic or inorganic molecules [1–5]. They are generated by an in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX), which was first reported in 1990 [6, 7]. The SELEX method has permitted the identification of unique RNA/DNA molecules from very large populations of random sequence oligomers (DNA or RNA libraries), and they bind to the target molecule with high affinity and specificity. Aptamers show a very high affinity for their targets, with dissociation constants typically from the micromolar to the low picomolar range, comparable to and sometimes even better than those of some monoclonal antibodies [8].

In comparison to antibodies, aptamer receptors have a number of advantages that make them very promising for analytical and diagnostic applications. The main advantage is overcoming the problem of using animals for their production. Aptamers are isolated by in vitro methods that do not require animals: an in vitro combinatorial library can be generated against any target. Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind a specific region of the target, with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-by-batch variation found when using antibodies and, by chemical synthesis, modifications in the aptamer can be introduced to enhance the stability, affinity, and specificity of the molecule. Finally, because of their simple structure, sensor layers based on aptamers can be regenerated more easily than antibody-based layers, are more resistant to

denaturation and have a much longer shelf life. Moreover, the selection process itself, with the amplification step, gives advantages to aptamers with respect to other "non-natural" receptors, such as oligopeptides, which cannot be amplified during their selection procedure. Due to all these characteristics, aptamers have been used in numerous investigations, as therapeutic [9] or diagnostic tools [10] and for the development of new drugs.

Dendritic cells (DCs) are professional antigen-presenting cells distributed throughout peripheral tissues to act as sentinels against invading pathogens [11]. Both maturation and migration of DCs are orchestrated carefully by panoplies of chemokines and adhesion molecules. Adhesion molecules are crucial for all cellular interactions of the DC during its journey from bone marrow into blood, from blood into the peripheral tissues and subsequently into lymphoid tissues. Recently, many new cell-surface molecules on DCs have been identified, and these may contribute to DC functions in controlling innate, as well as adaptive immunity. In particular, a large diversity of C-type lectins have been identified on DCs; some of these regulate pathogen recognition, while others regulate signaling or cellular interactions, such as DC migration and T cell binding. Several years ago, Geijtenbeek and his co-workers identified DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), a novel adhesion receptor on human DCs that is essential in several key functions throughout the DC lifecycle [12], especially in the interactions between DCs and T cells or vascular endothelial cells (ECs).

The human DC-SIGN gene is located on chromosome 19p13.3 [13]. Its length is 1.3 kb, and it contains seven extrons and six introns [14]. DC-SIGN (CD209) is a type II transmembrane protein that, based on its structure, belongs to the C-type lectin family [15]. It is composed of 404 amino acids, and its molecular weight is 44 kD [12]. DC-SIGN contains a short cytoplasmic N-terminal domain with several intracellular sorting motifs containing amino acid residues that regulate internalization, an extracellular stalk of seven complete and one partial tandem repeat that have been proposed to regulate multimerization, and a C-terminal lectin or carbohydrate-recognition domain that contains an EPN sequence which is postulated to recognize mannose-containing carbohydrates.

In this study, we aimed at generating aptamers against DC-SIGN to develop potential regulators of the immune response. In view of the recent advances in SELEX methodology and because of the numerous reports showing successful generation of aptamers against proteins, we decided to use the microwell plate screening technique and biotin–streptavidin magnesphere separation during the SELEX process to generate DNA aptamers against the DC-SIGN protein.

Materials and methods

DNA library and primers

The DNA oligonucleotide library contained a 35-base central random sequence flanked by primer sites on either side: 5′-CGGGGATCCGGAATTCTCCTCACA-N35-GTATGTCGACGAAGCTTGCG-3′. The forward primer (5′-CGGGGATCCGGAATTCTCCTCACA-3′) and the biotin-labeled reverse primer (5′-Bio-CGCAAGCTTCGTCGACATAC-3′) were used in PCR to obtain double-labeled DNA, and to separate the single-stranded DNA by streptavidin-coated magnetic beads (Promega Corporation, Madison, USA). The library and all primers were synthesized by Shanghai Shenyou Biotech (Shanghai, China). All four bases (A, T, C, and G) were represented at each of the degenerate positions and both the library and primers used for the PCR amplification were HPLC-purified.

SELEX procedure

Selecting DNA aptamers with a high affinity for recombinant human DC-SIGN/CD209/Fc Chimera (R&D Systems, USA) was performed as follows. DC-SIGN protein was coated on a 96-well ELISA plate with 0.05 mol/l $NaHCO_3$ (pH 9.6) at 4°C overnight. Then DC-SIGN-coated wells and blank wells were blocked by 3% BSA at 37°C. The ssDNA pools were denatured by heating at 90°C for 5 min in a binding buffer (SHCMK) containing 20 mmol/l Hepes, pH 7.35, 120 mmol/l KCl, 1 mmol/l $CaCl_2$, and 1 mmol/l $MgCl_2$ and then denatured at room temperature for 15 min. In order to reduce background binding, a 5-fold molar excess of yeast tRNA (Invitrogen, Karlsruhe, Germany) was added. The ssDNA library was combined in BSA-blocked blank wells at 37°C for 45 min, in order to screen out ssDNA, which had bound to BSA. Then unbound ssDNA was incubated with DC-SIGN-coated wells at 37°C for 40 min. Unbound ssDNA sequences were washed six times with washing buffer (SHCMK + 0.05% Tween 20). After that, eluting buffer (20 mmol/l Tris–HCl, 4 mol/l guanidinium isothiocyanate, 1 mmol/l DTT, pH 8.3) was added and incubated at 80°C for 10 min, and ssDNA bound to DC-SIGN was eluted and mixed with phenol-chloroform. The mixture was centrifuged at 12,000*g* for 5 min at 4°C, and the supernatant was mixed with dehydrated alcohol and 3 mol/l NaAc overnight at −20°C, then centrifuged at 12,000*g* for 20 min at 4°C. After the supernatant was removed, 75% alcohol was added to the sediment and centrifuged for 10 min. The precipitate was dissolved in 30 μl TE buffer (pH 8.0). DC-SIGN-bound ssDNA was amplified by PCR (Master Mix, Promega, Mannheim, Germany). Biotin-labeled primers

were used in PCR amplification (3 min at 94°C, then 40 s at 94°C, 1 min at 65°C, and 2 min at 72°C, followed by 7 min at 72°C). Streptavidin magnesphere paramagnetic particles (Promega, Madison, USA) were added to the PCR product to separate the ssDNA, which was used as the enriched library for the next selection round.

Cloning and sequencing of DNA aptamers

After 11 rounds of aptamer selection, the PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), then subcloned into a pMD 18-T vector with a TA cloning kit (TaKaRa, Dalian, China). Both purified PCR product and vector pMD 18-T were enzymatically digested with the restriction enzyme, EcoR V (Promega, USA). Different amounts of DNA and vector were ligated with T4 DNA ligase at 4°C. The resulting pMD 18-T derivatives carrying the 11 rounds of DNA sequences were transformed into $CaCl_2$-competent JM109 cells. Plasmids from individual bacterial clones were sequenced (Bioasia, Shanghai, China).

Measurement of the binding affinities of selected DNA aptamers

DNA aptamers were internally radiolabeled with [γ-^{32}P] ATP (Bioasia, Shanghai, China). 10 pmol of 5′end-labeled ssDNA from the library and various quantities of DC-SIGN protein were reacted in SHCMK buffer at 37°C for 40 min. The samples were vacuum filtered into nitrocellulose filters, then washed with SHCMK buffer. The dry nitrocellulose filters were put into scintillation discs containing 3 ml PPO-POPOP-dimethyl benzene. The binding affinity was measured in a Wallac WinSpectral 1414 lipid scintillation counter (Perkin–Elmer, MA, USA). The dissociation constant, K_d, was calculated by nonlinear regression analysis.

DC culture

DCs were prepared as previously described [16]. A white blood cell (WBC) suspension contributed by normal donors was obtained from the Blood Center of Zhejiang Province. The WBC suspension mixed at 1:1 with normal saline was layered over equal Ficoll and centrifuged at 1,300 g for 20 min at room temperature. Peripheral blood mononuclear cells were harvested from the interface and washed three times with saline. $CD14^+$ cells with >98% purity was separated by means of the immunomagnetic technique. Then those cells were plated in 6-well culture plates at a density of 2×10^5/ml in RPMI 1640 containing 10% FCS supplemented with rhGM-CSF (20 ng/ml) and rhIL-4 (20 ng/ml). Cultures were fed every other day by replacing half the medium. On day 5, buoyant cells were harvested as immature DCs (imDCs) for adhesion assay.

EC culture

Human ECs were obtained from umbilical veins and cultured as previously described [17]. Cords without HBV and HIV contaminants were contributed by normal parturients from Women's Hospital, Zhejiang University School of Medicine. Routinely, human umbilical vein endothelial cells were digested with 0.1% collagenase type I at 37°C for about 30 min. Then these cells were plated in 24-well plates at a density of 2×10^5/ml in M199 medium containing 15% FCS supplemented with EGF (10 ng/ml), 100 μg/ml penicillin, and 100 μg/ml streptomycin, and grown at 37°C in humidified 5% CO_2. The medium was refreshed every 2–3 days. The purity of EC cultures was checked by expression of von Willebrand (VIII) factor and found to be greater than 99% positive.

Adhesion assay [18]

ImDCs were incubated with 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, Molecular Probes, Invitrogen) twice for 30 min each, followed by washing to remove free dye, then with anti-DC-SIGN antibody (5 μg/ml, R&D, USA) or a specific aptamer (1 mmol/l) for 30 min. Labeled imDCs (2×10^5/ml) were added to EC monolayers which had been washed with Hanks balanced salt solution. After the two kinds of cells were coincubated for 1 h at 37°C, medium was aspirated and replaced with fresh PBS to remove nonadherent cells. Cells were fixed in 3.7% paraformaldehyde for 20 min. Adherent cells were immediately quantified using a Leica TCS SP Spectral Confocal Microscope (Germany). All adhesion experiments were performed with ≥ 3 different preparations of DCs. Endothelial adhesion of prestained DCs was analyzed in four independent high-power fields for each experiment.

Result

In vitro selection of aptamers for DC-SIGN protein

Human DC-SIGN protein was used as the target for *in vitro* selection of aptamers from a random pool of 10^{21} DNA molecules. The starting library was composed of 79 nt ssDNA containing randomized 35-nucleotide inserts.

About 12 rounds of selection were performed. In each round of selection, the concentration of competitor DNA was increased to further selection to create a small but high-affinity and high-specificity aptamer pool. The amount of DC-SIGN protein, ssDNA pool and tRNA, which were added in each round, and optimized cycles of PCR amplification are shown in Table 1. Especially, different cycles of PCR affected the quantity and specificity of the products. For example, after the 11th round of selection, 18, 20, 22, 23, 24, 25, 26, and 27 cycles of PCR amplification were performed, and the products were electrophoresed in 2% agarose. The products of 24 cycles of PCR amplification were relatively specific fragments (Fig. 1).

ssDNA pool binding to DC-SIGN protein

The ssDNA pools from rounds 1, 4, 7, and 11 were tested for their ability to bind to the DC-SIGN protein. The percentage binding of ssDNA to DC-SIGN protein increased from 0.54% in round 1 to 13.6% in round 4, 22.3% in round 7 and 31.7% in round 11. However, the value did not increase in the next round, indicating that the ssDNA, which specifically bound to DC-SIGN, had been maximally enriched after 11 rounds of selection.

Table 1 Selection parameters for DC-SIGN protein

SELEX rounds	DC-SIGN protein (μg)	ssDNA pool (pmol)	tRNA (mg/l)	PCR cycles
1	5	800	0	8
2	4	200	0.25	10
3	1	100	0.25	16
4	0.5	100	0.5	25
5	0.5	100	0.75	19
6	0.5	50	1	24
7	0.2	25	0.5	23
8	0.2	25	1	25
9	0.2	10	1	24
10	0.05	10	1	22
11	0.05	10	1	24

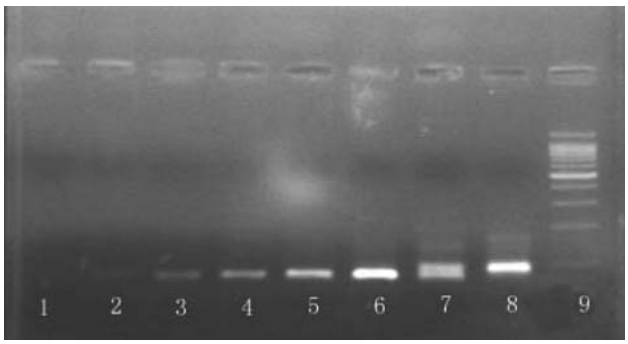

Fig. 1 dsDNA products by PCR after 11th round of selection. Electrophoresis on 2% agarose after 18–27 cycles of PCR amplification. Lanes 1–8: 18, 20, 22, 23, 24, 25, 26, and 27 cycles of PCR, lane 9: DL-1500 DNA Marker (TaKaRa)

Sequence analysis of 21 DNA aptamers

Material from round 11 selections were purified and cloned, and their inserts were analyzed. The alignment of consensus motifs was identified by inspection with the aid of computer-assisted search engines. The sequences of 21 clones from round 11 are shown in Table 2 (for each clone, only the variable 35 nt region without primer strands is shown).

Affinity of selected DNA aptamers

The binding affinity of the 21 selected aptamers was measured by lipid scintillation counter, except for 10z (only four bases). Among these aptamers, the values of DPM for 1, 6, 16, and 19 were high (Fig. 2a), so these four aptamers had higher affinity for DC-SIGN protein than the others. Then, the K_ds of these aptamers were measured (Fig. 2b). The K_d of aptamer 16 was 21.73 nmol/l, lower than the other three, meaning that this aptamer that had the highest affinity for DC-SIGN protein (Fig. 2c).

Effects of antibody and aptamer on DC adhesion to ECs

ImDCs pretreated with anti-DC-SIGN monoclonal antibody or aptamer 16 were co-incubated with EC monolayers for 1 h. Nonadherent cells were removed, and adherent cells were quantified using a confocal microscope (400×). CMFDA-labeled DCs were excited at 488 nm to express green light. Both antibody and aptamer blocked DC-EC adhesion (Fig. 3); they did not significantly differ.

Discussion

DCs, the sentinels of the immune system, are the first immune cells to come into contact with invading pathogens. A fundamental aspect of DC function in controlling immunity is the capacity to provide continuous surveillance for incoming foreign antigens and a prompt response to present the encountered antigens to T cells [11, 19]. Adhesion and migration are essential to this process because DCs have to travel from their "nursery" to the

Table 2 Sequence analysis of 21 DNA aptamers

Clone	Sequence of variable region
28	-----GGGCTCACAGACTTGGTTGGTAGCGGTGGGGTTG
22	-----GAACCGTTAGTGCTTTCCCTTAGTATAGGTTGGAG
26	----GGACTCTCTTAGCCGGAGCTACGTCAACAACGGGT
29	--GTAATATGTCGAACAGCGAACGTTTCCAGAAGGAA
7	--------CGGCAAGTGCAGGTGATAGGTCGCAGAGTACGAAT
11	-----AAACGTGGAGTGCGG--CAAGGGCAGGAGTGTTCAAG
8	------TCCCTGTTGAGGTGAGT-CGGGTGGGTGATTCTGCA
6	------TCCTAACGGTCTGCGTGCTATCTGAATAATTCCTT
15	------AGTCCTTGCCTGGGGGGACTATTAGT-ATTAATACG
19	-----AAGTTGCCATTACTAAGAGTATTCACG-ACTCTTGC
16	------GGCGAAAATTTGTGGATATAGAGGGTTACTCGGAT
18	------GGGCATGCCTCAGCATGTCTGACGTTAGTATTCGA
12	----GCCGCATGTATGGTGGAGCGTGATTGGACGGGGT
3	-----GTGGGAGGGTC-GGGAAGGTTGTAATC-GCGGTATAC
13	CGTGGGAGCGGAGTGTAGGCGATATTTGAGGAGAT
9	-----GAGCGTGGTGAGGGGGTTACTAGAGGGTGAGTATA
1	----TGATTTCATCATTTACTTCAGACCTTGCAGGTGAT
14	--------TACGCCGAGTAGGGTGTGATTTGGGGCTGGGAAAT
17	CACGTAGGTGAATGTTCAGCTTGACCGCGGGAGA
21	---GCGTGGCCCGGTCCGGGGAGCATCTGATCTGCCGA
20	------AGCTGGGATCACTATTCTCTATTATTTGTTTCCAT

The highlighted bases indicate consensus sequences

inflamed tissue, where they capture antigen, and subsequently to the lymph nodes, where T cells reside [20]. Cell–cell interactions between DCs and T cells or ECs are strictly coordinated events, regulated by chemokines and cytokines, together with the expression of adhesion molecules on DCs, T cells, and ECs.

DC-SIGN, a novel DC-specific adhesion receptor, binds with high affinity to the intercellular adhesion molecule (ICAM)-2 on ECs and ICAM-3 on T cells [12, 21]. DC-SIGN-mediated migration of precursor and immature DCs from blood into peripheral tissues involves four steps: (1) DC rolling, (2) rapid activation of DCs, (3) adhesion to endothelial ligands through activated integrins, and (4) diapedesis [22]. The interaction of DCs with the ECs lining the blood vessels is an important control point in egress. Besides having a function in the rolling of DCs along the blood vessel lining, DC-SIGN also mediates the adhesion of DCs to endothelium *via* ICAM-2 and their subsequent transendothelial migration.

During the interactions between the body and pathogens or tumors, the latter can escape immune surveillance and survive. The mechanism was related to suppression of DCs by DC-SIGN, which had been cloned from a placental library, through its capacity to bind the envelope glycoprotein gp120 of HIV-1 [23]. Then, it was found to bind the glycosylated envelopes of other viruses such as Ebola virus, hepatitis C virus, dengue virus, cytomegalovirus, HIV-1, measles virus, human herpesvirus 8, and the SARS coronavirus, as well as *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Streptococcus pneumoniae*, fungi, and some parasites and tumors [24–27].

It is known that, on one hand, DC-SIGN can mediate DC migration, antigen internalization and T cell activation, while on the other hand, it can be the target of pathogens or tumor cells, and this may lead to escape from immune surveillance or immune suppression. Vaccines and agents based on DC-SIGN could provide new means of treating and preventing certain immune disorders and inflammatory diseases.

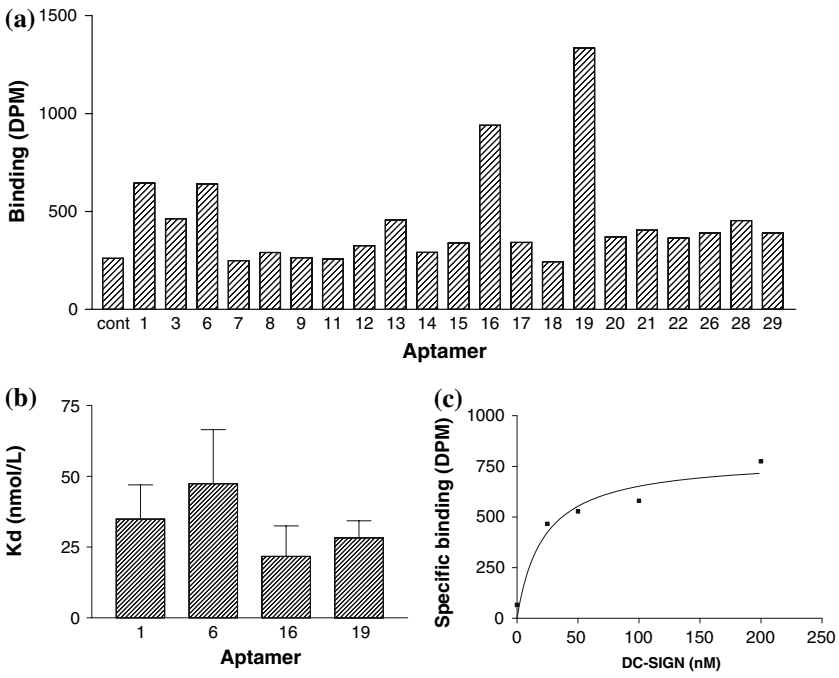

Fig. 2 Binding affinities of aptamers to DC-SIGN protein. (**a**) Binding affinities of 21 selected aptamers measured by lipid scintillation counter. Among these [γ-^{32}P]-labeled aptamers, the DPM values of 1, 6, 16, and 19 were relatively high (DPM represents the intensity of radioactivity). (**b**) K_ds of aptamers 1, 6, 16, and 19; that of aptamer 16 was lowest. Data presented as mean ± SE, $n = 3$. (**c**) Binding curve for aptamer 16

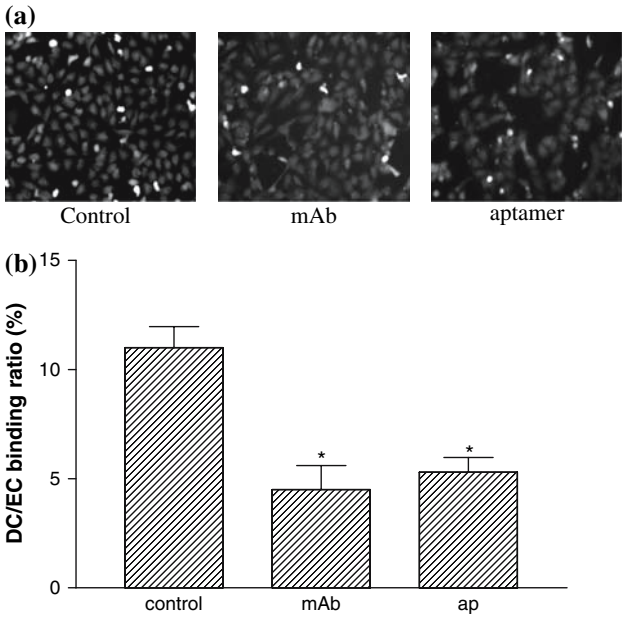

Fig. 3 Effects of anti-DC-SIGN monoclonal antibody and aptamer 16 on DC adhesion to Ecs. (**a**) Representative confocal microscope (400×) images of CHFDA-labeled DCs adherent to ECs without pretreatment (Control), and after pre-treatment with anti-DC-SIGN monoclonal antibody (mAb) or aptamer 16 (aptamer). (**b**) Numbers of DCs and ECs in an area of 5×10^4 μm^2 from images such as those in (**a**). Anti-DC-SIGN monoclonal antibody and aptamer blocked DC adhesion to ECs, and they did not significantly differ. Data presented as mean ± SE, $n = 6$. $^*P < 0.05$ versus control

In our study, we set out to select DNA aptamers to block DC-SIGN binding to ICAM-2 and ICAM-3 in order to interrupt the interaction between DCs and EC or T cells. The SELEX technique has been used to randomly select short RNAs, DNAs or peptides that selectively bind to a target protein. Aptamers are antibody analogs in terms of both specificity and affinity, with an apparent advantage over antibodies in that they can be produced by automated chemical synthesis. There are many reports about selection of aptamers against target proteins from a random oligonucleotide library. For example, Beinoraviciute–Kellner selected for specific ABF1 binding sequences on double-stranded DNA by a SELEX procedure in vitro [28]. Moreno designed a novel SELEX methodology using colloidal gold to select for high-affinity single-stranded DNA aptamers against *Leishmania infantum* KMP-11 [29].

Libraries in SELEX typically contain 10^{14}–10^{15} independent nucleic acid sequences. This number is small compared to the number of possible sequences, minimizing the probability of replicate sequences being present. For example, there are 10^{24} (i.e. 4^{40}) possible sequences in a library containing a 40-base random region. While it is not practical to create comprehensive libraries, large libraries do increase the probability that high-affinity ligands will be present. We constructed a DNA oligonucleotide library containing a 35-base central random sequence, and selected for ssDNA binding to DC-SIGN protein by using a microwell plate screening technique, PCR and biotin–streptavidin magnesphere separation during the SELEX process. After 11 rounds of selection, we cloned, purified, and sequenced the PCR products, and obtained 21 DNA aptamers against DC-SIGN. Although there were few consensus regions among all the sequences, more consensus regions were found by comparisons in subgroups. Since DC-SIGN is a large molecule, different sequences correspond to different sites, leading to great diversity in the aptamer pool.

The binding affinity of selected aptamers was measured by lipid scintillation counter, and the K_d value was calculated. This revealed that aptamer 16 had a higher binding affinity for the DC-SIGN protein, and the K_d value was 21.73 nmol/l.

Then, aptamer 16 was used in cytological experiments to assess its physiological function, and the adhesion assay was carried out. DCs were pretreated with anti-DC-SIGN monoclonal antibody or aptamer, before co-incubation with ECs. Both antibody and aptamer reduced the number of DCs adherent to ECs compared with the control, and there was no significant different between them. So, the effect of the DC-SIGN specific aptamer on inhibiting DC-EC adhesion was comparable to that of the monoclonal antibody. This effect was due to the aptamer binding to DC-SIGN on the DC surface and inactivating it.

In conclusion, we selected an aptamer with high affinity to DC-SIGN that blocked DC adhesion to ECs with an effectiveness comparable to the antibody. The sequence of this aptamer is 5′-GGCGAAAATTTGTGGATATAGAG GGTTACTCGGAT-3′. However, further investigation of the construction and function of the aptamer is needed in order to assess its potential in basic research, drug development, and clinical applications.

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