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Aptamer selection for the inhibition of cell adhesion with fibronectin as target

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Abstract—An affinity column immobilizing a decapeptide H_2N-RGDSPASSKP-CO_2H was used to select RGD-binding aptamers from a pool of 86-mer single-strand oligodeoxynucleotides (ODNs) containing a random 40-mer sequence. The enriched library thus obtained was further selected against adsorbed fibronectin and individual aptamers were monocloned in *E. coli* and sequenced to give a couple of highly homologous ODNs, which indeed inhibited fibronectin-integrin mediated cell adhesion.

SELEX (*S*ystematic *E*volution of *L*igands by *Ex*ponential Enrichment)[1–3] is a highly sophisticated oligonucleotide selection procedure that has been used to isolate and amplify, among random libraries, aptamers capable of binding to a particular target such as protein. Especially intriguing are functional aptamers,[4,5] which interfere with protein–protein interactions. In cases where the interaction-responsible particular residues (epitopes) on the protein surface are identified, aptamers may be selected against an epitope-containing small peptide as target.[5] The present work is concerned about fibronectin. This huge dimeric protein (~230 kDa for each subunit), normally found in extracellular matrices, is constituted of a series of repeating modules known as types I–III and has multiple adhesive sites for various substances including fibrin, heparin, collagen, and integrin (cell) (Fig. 1a).[6] Remarkably, the critical ligand for cell-surface integrins $\alpha_5\beta_1$, $\alpha_\gamma\beta_3$, and $\alpha_{IIb}\beta_3$ is a simple tripeptide Arg-Gly-Asp (RGD) motif.[7] The fibronectin-integrin interactions are responsible for such diverse cell activities as adhesion, morphology, signaling, growth, differentiation and multiplication, migration and translocation, and angiogenesis and transfer of tumor cells.[7] We report here that the aptamers selected indeed inhibit cell adhesion.

An initial pool of 86-mer single-strand oligodeoxynucleotides (ODNs) contains a random 40-mer sequence (N_{40}, in ~10^{15} different sequences) with flanking primer regions having fixed sequences at both ends.[8] This ODN library was passed through an agarose-gel column immobilizing (peptide-CONH-column)[9] a short RGD peptide H_2N-RGDSPASSKP-CO_2H (Sigma) whose sequence was a direct copy of the RGD region of fibronectin. Most of ODNs passed through the column when eluted with PBS(+) buffer (pH 7.2) containing 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. A small amount (<1%) of binding ODNs adsorbed on the column was recovered with Tris–acetate elution buffer (pH 5.8) containing 7 M urea and 3 mM EDTA, ethanol-precipitated, and amplified via asymmetric PCR.[10] The enriched library obtained after five such cycles (Fig. 1b) was passed through a peptide-free column as a negative selection and subjected to a second selection against adsorbed fibronectin in order to remove nonfunctional aptamers that bind to the RGD peptide but not to the protein. Thus, the library obtained as above was incubated in PBS(+) in a fibronectin-precoated 96-well plate (BD BioCoat™ Fibronectin Cellware) and the plate was washed with PBS(+). Aptamers which survived washing on the plate were detached with elution buffer and PCR-amplified as above. This incubation/

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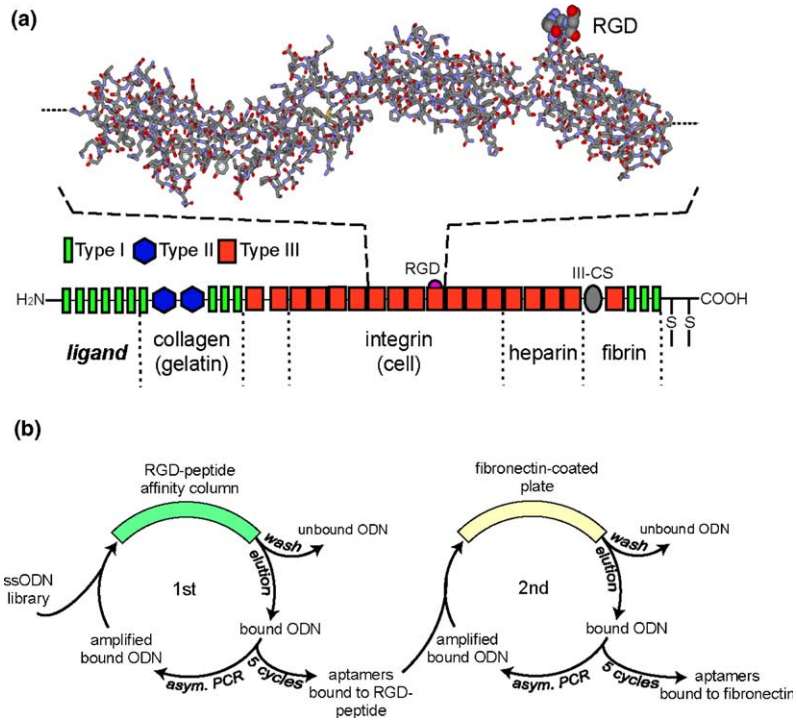

Figure 1. Illustration of fibronectin (subunit) structure with various adhesive domains (a) and sequential selection of random oligodeoxynucleotides (ODNs) based on the RGD peptide and the whole fibronectin protein (b).

washing/PCR cycle was repeated five times (Fig. 1b) under progressively severer conditions[11] to give a doubly selected ODN library.

Individual aptamers were monocloned in *E. coli* and seven aptamers arbitrarily chosen were sequenced as shown above. Among them, aptamers **1** and **2** are highly homologous with a difference only in one base. They are capable of strongly binding to fibronectin as revealed by gel shift [1.5% agarose gel containing ethidium bromide (1.0 μg/mL)] and SPR (surface plasmon resonance). Thus, aptamer **1** is readily adsorbed on a fibronectin-immobilized sensor chip,[12] while desorption thereof hardly takes place (Fig. 2).

We then moved on cell assay to see if the present RGD-selected, fibronectin-binding aptamer **1** can inhibit cell adhesion.[13] Baby hamster kidney (BHK) cells (Riken Cell Bank, accession no. RCB1423) were incubated in a fibronectin-precoated 96-well plate in the absence and presence (10 μM) of ODN (aptamer **1** or $d(A)_{86}$ as a control). After incubation for 2 h at 37 °C, the mixture was analyzed with an optical microscope for cell shapes and numbers of adherent/floating cells. Without any ODN or with control ODN $d(A)_{86}$, cells firmly adhere onto the plate with adhesion-induced characteristic elongation even after several washings (Fig. 3a and b).[14] In sharp contrast, elongation is completely suppressed in the presence of aptamer **1** (Fig. 3c); some cells appear to be weakly adhering on the plate but are thoroughly rinsed out upon washing (Fig. 3d). These results leave little doubt that aptamer **1** binds to fibronectin to inhibit the integrin-binding activity of the latter.[15]

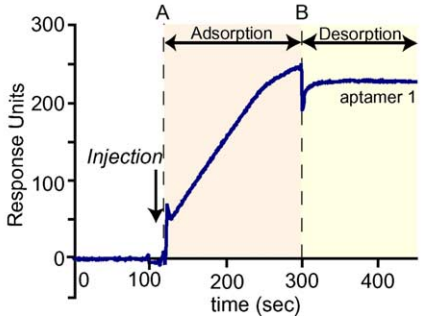

Figure 2. SPR trace of the interaction of aptamer **1** with immobilized fibronectin at 25 °C under flow (10 μL/min) of PBS(+) as running buffer. An aptamer solution (30 μL, 10 μM) in PBS(+) was injected at a flow rate of 10 μL/min for 180 s (from A to B). Response units were corrected for nonspecific interactions by using a fibronectin-free chip as a reference.

In summary, this work shows a SELEX route to cell-adhesion modulating fibronectin blockers.[16] The targeted RGD motif appears in a number of adhesive proteins. There are also many known examples of epitopes including antibody determinants. The essential theme of the present approach, while not yet clear, is the

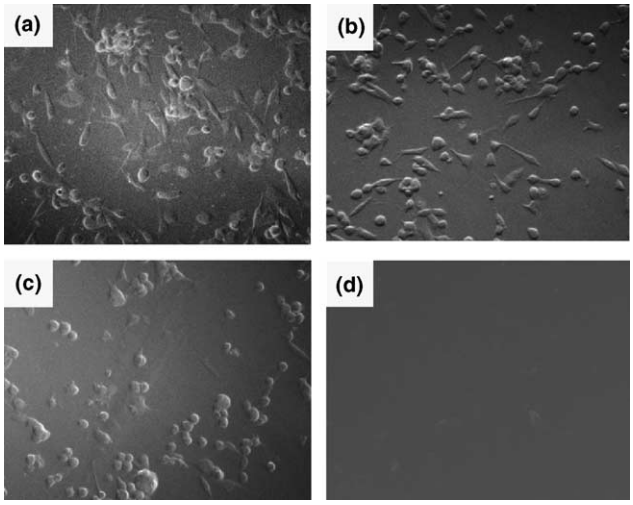

Figure 3. Optical microscopic images of BHK cells in PBS(+) buffer in a fibronectin-coated plate in the absence of any ODN (a) and in the presence of control ODN $d(A)_{86}$ (b) or aptamer **1** (c) without washing. Upon washing, cells in (a) and (b) resist to be detached, while those in (c) readily came off (d).

significance of the sequential two-step selection based first on the epitope peptide and then on the whole protein. Further work is now under way focusing on this point.

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8. The ODN library was prepared on an Applied Biosystems 392 DNA/RNA synthesizer, deprotected, and purified with Poly-pak II Cartridge (GREN RESEARCH).
9. Decapeptide was immobilized on an NHS-activated agarose gel column (Amersham, NHS = *N*-hydroxysuccinimide) according to the manufacturer's instruction to give an RGD-peptide affinity column.
10. Asymmetric PCR was carried out using PCR Master Mix Kit (Qiagen) with 6.7 μM 5′-primer and 0.2 μM 3′-primer. Each PCR consisted of 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1 min.
11. The conditions for the first cycle: incubation for 4 h in 100 μL of PBS(+) buffer (pH 7.2 with 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) followed by washing with PBS(+) (3 × 300 μL). Selection in subsequent cycles utilized severer conditions, that is, shorter incubation times (1.5, 0.5, 0.5, and 0.25 h for the second, third, fourth, and fifth cycles, respectively) with more washings (5 × 300 μL for the third, fourth, and fifth cycles).
12. Fibronectin (Sigma) was immobilized on a chip CM5 (carboxymethylated dextran, BIAcore) using an amine-coupling kit (BIAcore) under continuous flow (10 μL/min) of 10 mM HEPES buffer (pH 7.4) containing NaCl (150 mM). A solution (70 μL) of *N*-hydroxysuccinimide (50 mM) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (200 mM) was injected to activate the carboxymethylated dextran surface. A solution (100 μL) of fibronectin (1 mg/mL in aqueous sodium acetate, pH 5.0) was then injected onto the activated surface at a flow rate of 1 μL/min, followed by blocking with 70 μL of ethanolamine hydrochloride (1.0 M, pH 8.5) and washing with 10 × 10 μL of 50 mM NaOH to afford a fibronectin-coated CM5 chip (ΔRU, ~10,000).
13. A 50 μL PBS(+) solution of a cloned aptamer (10 μM) was incubated in a fibronectin-coated 96-well plate at 4 °C overnight. After removing the solution, the plate was washed with 2 × 100 μL of PBS(+) and then BHK cells were seeded on the plate at 37 °C. After incubation for 2 h, microscopic observation was made for the cell shape and numbers of adherent/floating cells in each well with an Olympus IX70 optical microscope.
14. No cell adsorption was detected when a fibronectin-free plate was used.

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