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Supplementary Information:

Evolutionary identification of affinity peptides for the detection of sepsis biomarker procalcitonin

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Materials and Methods

Chemicals

Recombinant human procalcitonin was purchased from Randox Life Science (Crumlin, UK). Horseradish peroxidase (HRP) conjugated anti-M13 monoclonal antibody was purchased from GE Healthcare (Piscataway, NJ). Tween 20 and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Sigma (St. Louis, MO). Biotin labeling kits (EZ-Link sulfo-NHS-Biotinylation kit), Coomassie protein assay kit, streptavidin coated microwell plates, and Zeba desalting spin columns were from Pierce Biotechnology (Rockford, IL). Unless otherwise stated, all of chemicals were of reagent grade.

E. coli strains and bacteriophages

Escherichia coli strain ER2738 as host for M13 phage infection and M13 random peptide library (Ph.D.-12) which expresses random and linear 12-mer peptides were obtained from New England Biolabs (Ipswich, MA). In brief, this peptide library is fused to pIII protein which causes five copies of a particular peptide to be displayed on the surface of phage and the sequence of each peptide is encoded in the phage genome. Phage particle purification, concentration and DNA isolation were carried out according to the instructions of the manufacturer. Clones of interest were sequenced using the -96 pIII sequencing primer (New England Biolabs, 5'-GCCCTCATAGTTAGCGTAACG-3').

Biotin labeling of recombinant human procalcitonin

Biotinylation of the procalcitonin proteins was carried out according to the instructions of the manufacturer. Briefly, procalcitonin was mixed with 10 mM of biotin solution (20-fold molar excess), and incubated on ice for 3 hrs. In order to remove non-reacted and hydrolyzed biotinylation reagents, the reaction mixtures were purified using a Zeba desalting spin column. To estimate the level of biotin incorporation, a solution containing the biotinylated procalcitonin was added to a HABA/avidin solution, and the absorbance of this solution at 500 nm was used to calculate the moles of biotin per mole of protein. Typical yields were about 85% labeled procalcitonin.

Evolutionary phage display (Biopanning)

Recombinant human procalcitonin were dissolved in 50 mM Tris-HCl (pH 7), 50 mM NaCl buffer and transferred to the wells of the 96-microwell plates. After overnight incubation at 4°C

with mild shaking, coated wells were filled with blocking buffer (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/mL BSA, 0.02 % NaN_3) and incubated at 4°C for 1 h. After removing the blocking solution, wells were washed six times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The Ph.D.-12 peptide library (1.5×10^{13} pfu) in 100 μL of TBS buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) was added to each well containing immobilized recombinant proteins, and the plate was shaken gently at 4°C for overnight. To remove unbound phages, the plate was washed 10 times with TBST. Five rounds of biopanning were performed for target protein. After washing, the bound phages were eluted using 100 μL of 0.2 M glycine–HCl (pH 2.2). The eluent was immediately neutralized with 15 μL Tris–HCl (pH 9.1) to prevent the destruction of the phage.

The eluted phages were amplified using *E. coli* ER2738 strains to make sufficient copies for subsequent rounds of biopanning. The amplified phages were harvested by NaCl/polyethylene glycol precipitation (20% (v/v) PEG-8000 with 2.5 M NaCl). After every round of biopanning, the recovered phage were titered by plating aliquots of the infected *E. coli* ER2738 prior to amplification on Luria–Bertani (LB) agar containing isopropyl β-D-thiogalactopyranoside (IPTG) and X-gal. The plates were incubated overnight at 37°C and the blue colonies were measured. The enrichment of bound phages was calculated as follows: output phage/input phage × 100. The blue plaques were randomly picked and amplified for DNA sequencing.

Sequence analysis

Single-stranded DNAs of positive phage clones were sequenced by Genotech (Daejeon, Korea) using the -96 pIII sequencing primer: 5′-CCC TCA TAG TTA GCG TAA CG-3′.

According to the phage pIII gene-derived reading frame in the coding strand, we derived the amino acid sequence of the exogenous protein fusion to the M13 coat protein pIII. BLAST searches were performed using the SWISSPROT database to determine sequence similarity with previously identified human peptides or proteins. The Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and ExPASy Tools (web.expasy.org/translate) translate were used to analyze the sequences identified earlier.

ELISA for peptide binding to procalcitonin

To test whether the selected phage clones could specifically bind to human recombinant procalcitonin, ELISA assays were performed. Plates were coated with recombinant proteins for overnight at 4°C, blocked with blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 1 % BSA, 0.02 % NaN_3), and washed six times with TBS solution. One hundred microliters of amplified phage (10^{11} pfu) was then added and incubated for 1 h at room temperature. After washing six times with the same buffer to remove unbound phage, HRP-conjugated anti-M13 monoclonal antibody (diluted 1:10,000 in blocking buffer) was added and incubated at room temperature for 1 h. The antibody solution was removed and the plate was washed again with TBS. Freshly prepared HRP substrate (36 μL of 30% H_2O_2 added to 21 mL of ABTS stock: 22 mg of 2,2'-Azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid in 100 mL of 50 mM sodium citrate, pH 4.0) was added and measured at 405 nm with a microplate spectrophotometer (Multiskan FC, Thermo Scientific, CA, USA). Polystyrene wells coated with 1 mg/mL of BSA, and wells incubated with 1 % of milk in TBS were used as negative controls.

Measurement of apparent dissociation constants

The measurement of apparent dissociation constants ($K_{d,\ app}$) for the selected peptides was performed according to the method of Friguet *et al.* (1985) with minor modifications. Briefly, recombinant procalcitonins at various concentrations were first incubated with the desired phage particles in TBS buffer containing 1% BSA at a fixed concentration (10^{11} pfu/mL). After 24 h of incubation at 4°C, 100 μl of each mixture were transferred into well plates pre-coated with each procalcitonin and re-incubated for 1 h at room temperature with mild shaking. After washing twice with TBS buffer, the bound phages were detected by adding the HRP-conjugated anti-M13 antibody and ABTS, and the color changes in the wells were measured at 405 nm. The apparent $K_{d,app}$ values were estimated from the slopes of the regression curves obtained by plotting the fraction of bound antibody versus the molar concentration of recombinant procalcitonin.

Table S1. Enrichments obtained during biopanning with the phage-displayed peptide library against human recombinant procalcitonin

Biopanning	Input	Output	Yield (%)	Tween 20 (%)
1	6.1×10^{10}	2.6×10^{5}	4.26×10^{-4}	0.1
2	8.0×10^{11}	5.5×10^{6}	6.87×10^{-4}	0.3
3	2.3×10^{11}	7.5×10^{6}	3.26×10^{-3}	0.5
4	8.0×10^{11}	4.9×10^{7}	6.12×10^{-3}	0.5
5	1.08×10^{11}	1.9×10^{7}	0.0175	0.5

[a] Percent yield was calculated as follows: output/input phage × 100

Table S2. Sequences of the selected phage clones following the 3th, 4th and 5th rounds of biopanning against human recombinant procalcitonin

Name	Amino acid	Frequency	Notes
3R#23	MSCAGHMCTRFV	1/24	Selected after 3th round
4R#22	QFDYMRPANDTH	1/24	Selected after 4th round
5R#24	AERVADHTVSVW	1/24	Selected after 5th round

Table S3. Analysis of the hydrophobicity/hydrophilicity of the selected peptides

Clones	Negative charged amino acid	Positive charged amino acid	Total net charge	Total hydrophobic ratio (%)
3R#23	MSCAGHMCTRFV	Histidine at position 6, Arginine at position 10	+1	58
4R#22	QFDYMRPANDTH	Arginine at position 6, Histidine at position 12	-1	25
5R#24	AERVADHTVSVW	Arginine at position 3, Histidine at position 7	-1	50

Sequence analysis was performed with secondary structure prediction program (http://aps.unmc.edu/AP/prediction/prediction_main.php)

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