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Identification of Peptides Inhibiting Adhesion of Monocytes to the Injured Vascular Endothelial Cells through Phage-displaying Screening

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Abstract Using oxidized low-density lipoprotein (LDL)-injured vascular endothelial cells (ECs) as target cells, peptides specifically binding to the injured ECs were screened from a phage-displaying peptide library by using the whole-cell screening technique after three cycles of the “adsorption-elution-amplification” procedure. Positive phage clones were identified by ELISA, and the inserted amino acid sequences in the displaying peptides were deduced from confirmation with DNA sequencing. The adhesion rate of ECs to monocytes was evaluated by cell counting. The activity of endothelial nitric oxide synthase (eNOS), and the expression levels of caveolin-1 and intercellular adhesion molecule-1 (ICAM-1) were determined by Western blotting. Six positive clones specifically binding to injured ECV304 endothelial cells were selected from fourteen clones. Interestingly, four phages had peptides with tandem leucine, and two of these even shared an identical sequence. Functional analysis demonstrated that the YCPRYVRRKLENELLVL peptide shared by two clones inhibited the expression of ICAM-1, increased nitric oxide concentration in the culture media, and upregulated the expression of caveolin-1 and eNOS. As a result, the adhesion rate of monocytes to ECV304 cells was significantly reduced by 12.1%. These data suggest that the anti-adhesion effect of these novel peptides is related to the regulation of the caveolin-1/nitric oxide signal transduction pathway, and could be of use in potential therapeutic agents against certain cardiovascular diseases initiated by vascular endothelial cell damage.

Key words random peptide phage library; vascular endothelial cell; whole-cell screening; cell adhesion; caveolin-1; eNOS

Vascular endothelial cell (EC) injury is the initial event in a variety of cardiovascular diseases, of which atherosclerosis is one. Increased monocyte adhesion to the endothelium has been observed in the early stages of atherosclerosis and other inflammatory diseases [1]. Oxidized LDL (ox-LDL), one of the major injuring agents, can damage human vascular endothelial cells and alter the expression of many proteins, including intercellular adhesion molecule-1 (ICAM-1), which is an important adhesion molecule in atherogenesis [2]. The injured endothelial cells may express specific proteins or have an altered cell-surface conformation. Recently, several ligands targeting various cells or organs have been identified using phage display technology [3–5]. For this reason, the altered surface architecture of injured endothelial cells could be a useful target in the development of new approaches for studying and treating certain cardiovascular diseases.

Phage display has been proven to be a powerful technique for the rapid selection and isolation of antibodies to any given target antigen [6,7]. Therefore, we utilized the phage display technique to screen for peptides that could bind to the surface of injured endothelial cells with high affinity. A 15-mer peptide phage display library was

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screened. We identified some positive phage clones that not only bound specifically to the cell surface of injured ECs but also possessed obvious anti-adhesion effects with respect to the interaction between monocytes and ECs.

Materials and Methods

Materials

We used a random library of 15-mer peptide f88.4 phages with tetracycline resistance (XCX15: C is cysteine and X represents one random amino acid) residing in an *E. coli* K91/Kan host strain. The phage complexity was 1.0×10^9. The fuse5 (both f88.4 and fuse5 belong to filamentous fd phage vectors) is a phage without exogenous peptides. The phage display library, the *E. coli* K91/Kan and the fuse5 phage were provided by Dr. Jia-Da LI [8]. The primer for sequencing (5′-CAACCACCA-TAGCCCAAG-3′) was synthesized by Sangon (Shanghai, China).

The endothelial cell line (ECV304) and human monocytic cell line (THP-1) were purchased from the China Center for Type Culture Collection, Wuhan University. Probucol (p.9672), which produced an inhibitive effect on the ox-LDL-induced adhesion of monocytes to endothelial cells *in vitro* [9], was purchased from Sigma (St. Louis, USA). The anti-M13 polyclonal antibody from the Institute of Biochemistry and Cell Biology (Shanghai, China) was derived from rabbit immunized with highly purified M13 bacterial phage particles [8]. Polyclonal anti-caveolin-1 (sc-884), endothelial nitric-oxide synthase (eNOS; sc-8311), anti-ICAM-1 (sc-1511) antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and Western blotting luminol reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from HyClone Pierce (USA). The ox-LDL was the product of the Department of Biochemistry, the Institute of Beijing Senior Hospital (China). The nitric oxide kit was purchased from the Jiancheng Institute of Biotechnology (Nanjing, China).

Selection of phages specifically binding to injured ECV304 cells by whole cell screening

The ECV304 cells were cultured in 24-well tissue culture plates with 500 μl RPMI 1640 media (Roswell Park Memorial Institute). When the cultures reached approximately 90% confluence, they were treated with ox-LDL (100 μg/ml) at 37 °C for 3 h and then exposed to approximately 1.0×10^{10} phages (10 μl of the original peptide library). After 1 h incubation at room temperature, the plates were washed six times with PBS to remove the unbound and nonspecific binding components. The bound phages were eluted with 500 μl of 0.1 M HCl-glycine buffer (pH 2.2), and the eluate was immediately neutralized with 80 μl of 1 M Tris buffer (pH 9.0). The titer of phage in each fraction was determined by a plaque assay. Phage eluates were amplified by infecting *E. coli* K91/Kan in liquid Luria-Bertani (LB) agar containing 20 μg/ml tetracycline, shaken at 37 °C for 24 h, and finally the eluates were purified by precipitation with polyethylene glycol and resuspended in 500 μl Tris-buffered saline (TBS) buffer. Aliquots (50 μl each) of purified phage were subsequently reapplied to newly injured ECV304 cells for the next round of screening. Individual phage clones from the third round screening were tested by using an enzyme-linked immunosorbent assay (ELISA).

Identification of phages specifically binding to injured ECV304 cells by ELISA

The ECV304 cells were cultured to approximately 90% confluence in 96-well tissue culture plates in 200 μl RPMI 1640 media after treatment with ox-LDL (100 μg/ml) at 37 °C for 4 h [10]. The plates were then washed with PBS to remove the culture media, then blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min. Individual clones with 2.5×10^{10} phage particles were added to each well and allowed to bind to the injured ECs during gentle shaking at room temperature for 1 h. Unbound phages were removed by six washes with Tris-buffered saline with Tween-20 (TBST). Bound phages were fixed to the injured ECs by addition of paraformaldehyde (3.7%) for 10 min, then the cells were washed and incubated in a blocking buffer for 30 min. The bound phages were assayed after incubation with 150 μl rabbit anti-M13 IgG (1:2000) for 1 h. After gently washing the plate six times with TBST, 150 μl HRP-conjugated goat anti-rabbit IgG (1:5000) was added, then the plates were incubated for 45 min (with gentle shaking). The plates were washed six times again with TBST, and then incubated for 30 min with tetramethylbenzidine (TMB) substrate buffer. The reaction was stopped with 2 M H_2SO_4. Absorbance at 450 nm (A_{450}) was determined by using an automated reader. The more the phage binds to ECs, the higher is the absorbance value at 450 nm . When the absorbance of phages bound to the injured ECV304 cells was twice as that of phages bound to the control ECV304 cells, the phage was considered to be a positive clone [11]. The fuse5 phages without exogenous peptide were used as the phage negative controls.

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DNA sequencing

After ELISA identification, positive clones were added to the *E. coli* K91/Kan culture for the amplification, purification and preparation of DNA. The DNA inserted into the positive clones was sequenced by Sangon (Shanghai, China) using an automatic DNA sequencer.

Detection of the anti-adhesion effects of positive clones

Monocytic THP-1 cells at a concentration of 1.4×10^4 cells/ml and either the individual positive phage clones, or the negative control phages (1.0×10^{10}), were added to ECV304 cells injured by ox-LDL. The tissue culture plates were then placed on a platform shaker and rotated at 100 *g* at 37 °C for 1 h. The culture media (containing non-adherent THP-1 cells) was removed from the plates and the non-adherent THP-1 cells were counted. The monocyte adhesion was determined by using the formula below:

$$\text{Adhesion rate} = \frac{[\text{THP1}]_{\text{total}} - [\text{THP1}]_{\text{free}}}{[\text{THP1}]_{\text{total}}} \times 100\%$$

in which, the Adhesion rate is the monocyte adhesion expressed in percent (%), $[\text{THP1}]_{\text{total}}$ is the number of the total THP-1 cells added, and $[\text{THP1}]_{\text{free}}$ is the number of the non-adherent THP-1 cells.

Western blotting analysis of the anti-adhesion mechanism of positive clones

The confluent ECV304 cells were exposed to ox-LDL (100 µg/ml), ox-LDL+probucol (40 µM), ox-LDL+fuse5, or ox-LDL+No. 10 phage at 37 °C for 4 h. The supernatants from cultured ECV304 cells were collected to determine the nitric oxide (NO) concentration according to procedures described in NO detection kit. Cell lysates were prepared by using an ice-cold lysis buffer [50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol]. The protein concentration of the lysates was determined with a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, USA). Total protein (40 µg) from each sample was subjected to 10% SDS-PAGE electrophoresis. Proteins were transferred to the polyvinylidene difluoride (PVDF) membranes using a wet blotting apparatus (Bio-Rad Laboratories, Hercules, USA). The primary and secondary antibodies were diluted with TBST containing 5% skimmed milk. After 1 h of incubation with the appropriate primary antibody (1:1000 polyclonal rabbit anti-caveolin-1, 1:1000 polyclonal goat anti-ICAM-1 or 1:800 polyclonal mouse anti-eNOS), membranes were washed four times (for 15 min each time) in TBST and the appropriate secondary peroxidase-conjugated antibody (goat anti-rabbit, rabbit anti-goat or rabbit anti-mouse; 1:5000) was added for another hour. Finally, the blots were visualized using the luminol reagent (Santa Cruz, USA). Densitometric analysis was performed using an imager and densitometer. The relative expression levels of ICAM-1, caveolin-1 and eNOS were measured by using densitometry scans of autoradiograms in the linear range of film development. The results of each experiment were normalized to the density of the control group, which was arbitrarily adjusted to 1.0 [12].

Statistical analysis

Data are given as mean±SD. A value of $P<0.05$ was considered statistically significant using the Student's *t* test.

Results

Enrichment of phage target by three rounds of screening

Transforming efficiency (TU) was evaluated by counting the number of clones in plaques of 1 ml phage input or elution. As shown in **Table 1**, a large proportion of the input phages were screened out by each round of the selection procedure, and both the second and the third round were significantly more efficient than the first round. The most dramatic enrichment occurred during the second

Table 1 Enrichment of ECV304 binding phages containing 15-mer peptides

Round of screening	Phage input (TU)	Phages eluted (TU)	Enrichment (%)
1st	2×10^8	4×10^4	2×10^{-4}
2nd	2×10^6	8×10^5	4×10^{-1}
3rd	1×10^7	2×10^6	2×10^{-1}

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round. These results demonstrate that the 15-mer phage displaying library peptides can bind specifically to the injured ECV304 cells and can be enriched through the repeated selection.

Purification of positive clones by phage ELISA

Fourteen clones randomly selected from the third round of screening of phage f88.4 were tested for binding to the injured ECV304 cells by using phage ELISA. Among them, six clones (No. 3, 7, 8, 10, 13 and 14) were obviously able to bind to the injured ECV304 cells in comparison with the control ECV304 cells (**Fig. 1**). In contrast, fuse5 phages bound to neither the control nor the injured ECV304 cells.

Sequence determination

DNA of the above six positive clones was extracted and subjected to DNA sequencing analyses. The inserted amino acid sequences were then deduced. Among them, four phages (No. 3, 7, 10 and 13) contained a tandem leucine, and two of these four clones (No. 3 and 10) contained the amino acid sequence YCPRYVRRKLENELLVL (**Table 2**).

Anti-adhesion activity analysis

These six positive clones were further tested for their anti-adhesion effects (**Table 3**). In comparison with the fuse5 phage group, clones 3 and 10 significantly decreased the percentage of ECs-monocyte adhesion from 35.4% to 23.6% and 23.0%, respectively, figures that are lower

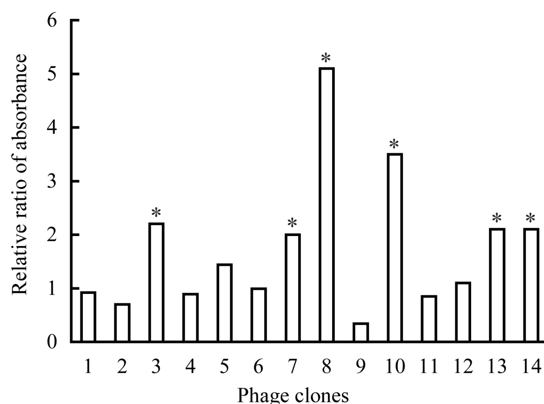

Fig. 1 ELISA identification of positive phage clones
Fourteen clones randomly selected from the third screening eluates were subjected to ELISA analysis. Absorbance at 450 nm was measured by using an automatic spectrometer, and the absorbance ratio of phage binding to injured ECV304 cells versus control ECV304 cells was calculated. Positive phage clones were designated as those with an absorbance ratio of equal to or higher than 2. * represents positive clones which bind specifically to the injured ECV304 cells.

Table 3 Anti-adhesion effect of six positive clones selected by phage ELISA

Group	Rate (%)
ox-LDL	40.6±1.7
Control	24.2±2.2 [aa]
ox-LDL+Probucol	27.3±3.6 [a]
ox-LDL+fuse5	35.4±2.8
ox-LDL+No. 3	23.6±3.0 [aa,b]
ox-LDL+No. 7	30.5±2.7 [a]
ox-LDL+No. 8	36.4±3.2
ox-LDL+No. 10	23.0±3.1 [aa,b]
ox-LDL+No. 13	27.3±1.8 [a,b]
ox-LDL+No. 14	31.8±0.9 [a]

[a] $P<0.05$, [aa] $P<0.01$ vs. ox-LDL group; [b] $P<0.05$ vs. ox-LDL+fuse5 group; $n=6$.

Table 2 Partial amino acid sequence of six positive clones deduced from direct DNA sequencing

Clone	Sequence
Phage No. 3	**NH_2-YCPRYVRRKLENELLVL-COOH**
Phage No. 7	NH_2-PCLMYSKYNLLYPYPAY-COOH
Phage No. 8	NH_2-GCMMYRYPAPGNHHIQM-COOH
Phage No. 10	**NH_2-YCPRYVRRKLENELLVL-COOH**
Phage No. 13	NH_2-RCCTRKRFLRWYGHSLL-COOH
Phage No. 14	NH_2-SCHFEALRRQTYMYFYV-COOH

Phage No. 3, 7, 10 and 13 contained a tandem leucine (LL, underlined), and phage No. 3 and 10 contained the same amino acid sequence (in bold).

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than that of positive control (27.3%), which was treated with probucol, an antioxidant with an effect against cell adhesion [9].

Effects of phage-binding on cellular gene expression

To further investigate the anti-adhesion mechanism of phage clone No. 10, we examined the expression of ICAM-1, caveolin-1 and eNOS in the ECV304 cells by using Western blotting (**Fig. 2**). We found that treatment with ox-LDL (100 μg/ml) for 4 h significantly increased the expression of ICAM-1 and decreased the expression of caveolin-1 and eNOS in cultured ECV304 cells. Pretreatment with probucol or phage clone No. 10 significantly inhibited the effect of ox-LDL on the expression of ICAM-1, caveolin-1 and eNOS compared with the ox-LDL treated group. However, fuse5 pretreatment did not affect the effect of ox-LDL on the expression of ICAM-1, caveolin-1 or eNOS.

As shown in **Fig. 3**, the NO concentration in the conditioned media of ox-LDL treated ECV304 cells decreased from 50.47±5.35 μM to 16.25±2.38 μM compared with the control. There was no significant difference between the ox-LDL plus fuse5 group and the ox-LDL group. Both the probucol positive control and the peptides from the positive phage clone No. 10 we identified significantly enhanced the functional recovery of injured endothelial cells by increasing the release of NO (35.80±5.33 and 26.89±4.21 vs. 16.25±2.38 μM, respectively).

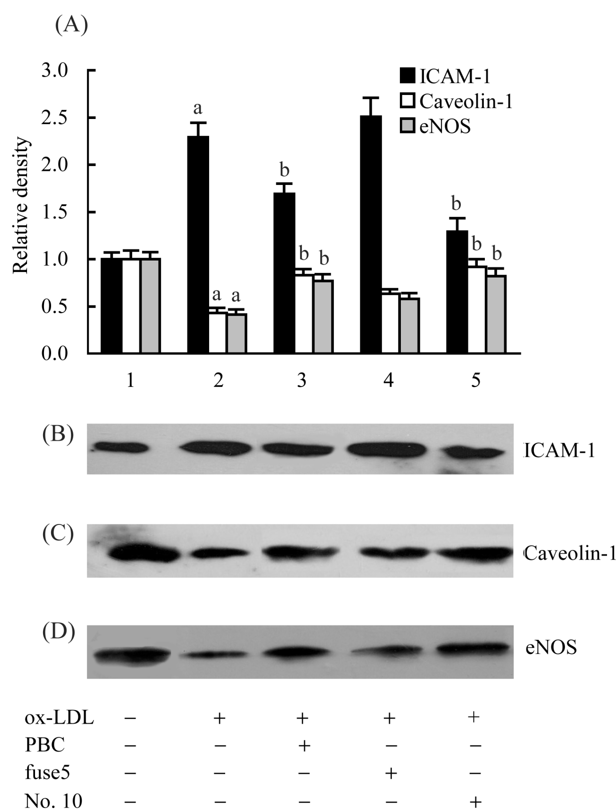

Fig. 2 Effect of phage peptide and probucol on the expression of ICAM-1, caveolin-1 and eNOS induced by ox-LDL

(A) The relative expressions of ICAM-1, caveolin-1 and eNOS were measured by densitometry scans of autoradiograms in the linear range of film development. The result of each experiment was normalized to the density of the control group, which was arbitrarily adjusted to 1.0. Results were expressed as the mean±SD of three experiments. [a] $P<0.01$ vs. the control (group 1); [b] $P<0.05$ vs. ox-LDL group (group 2). (B–D) Representative Western blotting of ICAM-1, caveolin-1 and eNOS, respectively.

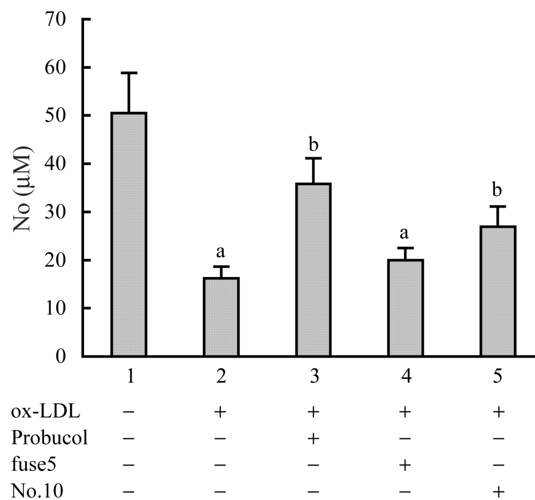

Fig. 3 Effects of phage peptide and probucol on NO release of ECV304 cells injured by ox-LDL

All data are mean±SD of six independent experiments. a, $P<0.01$ compared with the control (group 1); b, $P<0.05$ compared with the ox-LDL group (group 2).

Discussion

Phage display is a powerful technology for selecting and engineering peptides. The advantages of phage display technology over other research tools, and its great potential, have been demonstrated by its successful application in a variety of biomedical and clinical studies [13−15]. In the present study, we identified positive phage clones by screening a phage display library using injured ECV304 cells as the target. From a random 15-mer peptide f88.4 phage display library, we isolated six positive phage clones

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specifically binding to ox-LDL injured ECV304 cells. Some of them had a significant inhibitory effect on the adhesion of injured ECV304 cells to THP-1 cells. Interestingly, some of the phage clones with anti-adhesive properties (No. 3, 7, 10 and 13) possessed tandem leucines (LL), and some contained the RY motif (No. 3, 8 and 10). The LLG motif is broadly present on the intercellular adhesion molecule ICAM-1, the major β-2 integrin ligand, and several matrix proteins including the von Willebrand factor [16]. Arg-Gly-Asp (RGD) is a unique minimal integrin-binding sequence that was found within several glycoprotein ligands. An RGD-containing peptide has been shown to play a key role in integrin-mediated cell adhesion [17]. Wattam *et al.* found that when the motifs RYD and RCD were introduced into the dendroaspin scaffold to replace RGD, the RYD motif produced a similar IC_{50} value to the RGD motif in inhibiting A375-SM cell (β-3 integrin) adhesion to collagen [18]. In addition, the effect of LL in tandem on anti-adhesion may be related to its lipotropic nature. Further studies will be needed to confirm this.

Since clones No. 3 and 10 had the strongest anti-adhesion properties and shared an identical peptide sequence, we further investigated the anti-adhesion mechanisms of the peptide of clone No. 10. Western blotting analysis indicated that this peptide inhibited the expression of ICAM-1, up-regulated the expression of caveolin-1, enhanced the activity of eNOS and increased NO concentration, suggesting a role for these gene products in ox-LDL induced cell injury. Other investigators also found that the antioxidant probucol inhibits the ox-LDL induced adhesion of monocytes to endothelial cells *in vitro*, and produces similar alterations in gene expression to those found in our study (of the peptide of clone No. 10) [9].

ICAM-1 is an important adhesion molecule in atherogenesis. The high expression level of ICAM-1 in endothelial cells is stimulated by ox-LDL [19,20]. NO can inhibit the expression of ICAM-1 by regulating NF-κB [21]. NO, a product of eNOS, is a relaxing factor derived from the endothelium. eNOS activity is regulated by a variety of factors, including acylation by its cellular localization, and protein-protein interaction (i.e. eNOS-caveolin-1) [22]. It has been reported that eNOS is located in the caveolae, specialized invaginations on the plasma membrane of endothelial cells [23]. The main components of caveolae are cholesterol, glycosphingolipids, and some structural proteins, such as caveolin [24]. ox-LDL depletes cholesterol in the caveolae, resulting in the displacement of endothelial nitric oxide synthase from caveolae and impairment of eNOS activation [25]. eNOS binds to caveolin-1 in the lipid domain of the caveolae, and caveolin-1, as a protein chaperone of eNOS, regulates nitric oxide synthesis localization and activation in endothelial cells [22]. Therefore, the binding of the clone No. 10 phage peptide to the injured ECV304 cells may inhibit ICAM-1 expression by upregulating eNOS and caveolin-1 and increase the production of NO, leading to inhibition of adhesion to the monocytes.

In conclusion, our experimental data provide convincing evidence that our positive phage clones have peptides that specifically bind to the injured ECV304 cells. Furthermore, the anti-adhesion function of the clone No. 10 phage peptide may be related to the regulation of the caveolin-1/NO signal transduction pathway. Further studies need to be performed to analyze the crucial role of this signal pathway in ox-LDL induced endothelial injury and to evaluate the potential application of these peptides in the diagnosis and treatment of atherosclerosis.

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