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Phage-display library biopanning and bioinformatic analysis yielded a high-affinity peptide to inflamed vascular endothelium both *in vitro* and *in vivo*

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ABSTRACT

Vascular inflammation is considered the primary pathological condition occurring in many chronic diseases. To detect the inflamed endothelium via imaging analysis or guide the drug to target lesions is therefore important for early diagnosis and treatment of vascular inflammatory diseases. In this study, we obtained a novel peptide NTTTH through high throughout biopanning and bioinformatic analysis. *In vitro* studies indicated that NTTTH homologs could especially target inflamed vascular endothelial cells, as imaging quantitative analysis indicated that the mean of integrated optical density (MIOD) and mean of stained area (MSA) were significantly higher versus control ($P < 0.05$). In vivo studies showed that, after intravenous injection of enhanced green fluorescent protein (EGFP)-labeled NTTTH homologs into the lipopolysaccharide (LPS)-inflamed mice for 30 min, NTTTH homologs were distributed in highly vascularized and inflamed organs like liver and kidney. As a control, little fluorescence could be detected in mice injected with EGFP alone. Cryosection showed that NTTTH homologs especially targeted inflamed vasculatures but not normal ones. We did not detect fluorescence signal in either normal or inflamed mice which were injected with EGFP alone. The results suggested the role of NTTTH homologs in guiding the targeted binding of EGFP to inflamed vasculature and the potential usage for imaging detection and drug delivery.

1. Introduction

Vascular inflammation describes a systemic pathological state associated with several disease processes, including coagulation, neovascularization, ischemia and hypoxia, as occurs in many chronic diseases like cancers [1,2], systemic lupus erythematosus [3–5], rheumatoid arthritis [6], diabetes [7,8] and atherosclerosis [9]. Endothelism plays an important role in the development of these chronic diseases. Under normal state, endothelial cells function as the major regulator of vascular homeostasis. Endothelial cells maintain the balance between vasodilation and vasoconstriction, fibrinolytics and anti-fibrinolytics, inflammation and anti-inflammation, through releasing numerous vasodilator and vasoconstrictor molecules, procoagulants and anti-coagulants, inflammatory and anti-inflammatory cytokines, fibrinolytics and anti-fibrinolytics, oxidizing and anti-oxidizing molecules [10]. However, the balance will be upset when endothelium is injured under inflammatory conditions, resulting in endothelial hyper-permeability, leukocyte aggregation, platelet adhesion, and secretion of cytokines, and finally leading to the further development of inflammation and vascular diseases [11].

Obviously, the protein profile expressed in normal endothelial cells differs a lot from that in inflamed cells [12–15]. Under the inflammatory state, some molecules on the cell surface change in structure to expose its binding site, or up- or down-regulate its expression level. Theoretically, these molecules which change in type, amount and structure in response to the inflammatory stimulation are functionally associated with vascular inflammation, as receptors or ligands activated to mediate pro-inflammatory signals into target cells. Therefore, screening and identification of the differential proteins under inflammation is important for exploring novel inflammatory mechanisms and anti-inflammatory targets. On the other hand, the ligand molecule of these differential proteins is also of great interest. Exploring the ligand molecule of differential proteins is difficult but promising. Firstly, the ligand of these differential proteins could competitively inhibit the binding of pro-inflammatory mediators to effector cells; secondly, the ligand of these differential proteins could function as a guiding peptide for imaging detection and drug delivery. Phage display technology made this theoretical possibility into reality [16,17]. Since 1985 when phage display was first described [18], several novel anti-

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inflammation drug targets and active peptides identified using phage display technology have been reported [19–21]. However, the potential drug targets in inflamed endothelism are still far from comprehensively known. Moreover, it is still a challenge to monitor the inflamed endothelium in pathological tissues like atherosclerotic plaque, synovial tissues in rheumatoid arthritis and lesions in vital organs for early diagnosis and drug delivery.

In this study, we obtained a high-affinity peptide to inflamed vascular endothelium, NTSTH, through high throughout phage-display library biopanning and bioinformatic analysis. Though NTSTH does not naturally exist in known proteins, an on-line basic local alignment search tool (BLAST) indicated that there exist 2 homologous proteins, Scube1 and Scube2, both of which carry the homologous motif NTTTH at the C terminal and are functionally associated with endothelium inflammation [22–25]. We presumed that NTTTH and its homologous motif have targeted binding to inflamed endothelium and confirmed this *in vivo* and in vitro. The result showed that NTTTH homologs could especially target inflamed endothelium, and hold promise as a guiding peptide for imaging detection as well as drug delivery.

2. Materials and methods

2.1. Cells

Human umbilical vein endothelial cells (HUVEC) were harvested by collagenase (1 mg/ml) treatment from freshly collected umbilical cords of anonymized health donors from the Obstetrics and Gynecology Department, Nanfang Hospital. Human dermal microvascular endothelial cells (HMVEC) were kindly offered by Dr. Zhaoming, Southern Medical University. Both HUVEC and HMVEC were cultured on collagen-coated culture dishes. HUVEC were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, US HyClone) supplemented with 20% fetal bovine serum (New England Biolabs), 100 μg/ml cell growth factor (Invitrogen), 2.0 μmol/ml L-glutamine (Gibco-BRL) and 100 U/ml mycillin (New England Biolabs). HMVEC were cultured in MCDB-131 medium (New England Biolabs) with the same supplements as those in RPMI-1640. The cells were cultured under standard conditions at 37 °C in a 5% CO_2-humidified atmosphere. All the cells were passaged no more than five times before use.

2.2. Biopanning assay

HUVEC were grown to confluence then serum-starved in RPMI 1640 for 12 h. HUVEC induced by 100 ng/ml lipopolysaccharides (LPS, containing 100 ng/ml re-combinant human CD14, Sigma) for 12 h were used as bait, and HUVEC without induction of LPS were used as controls to remove false positive results. After 12 h of incubation with RPMI 1640, HUVEC were harvested with 0.25% pancreatin and centrifuged at 1000 rpm for 5 min. Then the cells were resuspended in RPMI 1640 containing 1% bovine serum albumin (BSA, New England Biolabs) with a density of 5×10^6 cell/ml. 200 μl suspended cells were transferred to a 1.5 ml Eppendorf tube, then added with 10 μl C7C phage library (1.0×10^{11} pfu, New England Biolabs) and slowly pendulated on ice for 2 h. After that, the incubated cells were transferred to the top of another Eppendorf tube containing 200 μl organic mixture (dibutyl phthalate:cyclohexane = 9:1, v/v) and centrifuged at 10,000 rpm for 10 min. The non-binding phages were left in the supernatant. The inflamed cells were harvested with 0.25% pancreatin and centrifuged at 1000 rpm for 5 min, then resuspended in RPMI 1640 containing 1% BSA with a density of 1×10^7 cell/ml. 100 μl suspended inflamed cells were transferred to a new 1.5 ml Eppendorf tube, then added with 200 μl of the top-layer phages and slowly pendulated on ice for 4 h. After that, 300 μl of the incubated cells were transferred to the top of another Eppendorf tube containing 300 μl organic mixture (dibutyl phthalate: cyclohexane = 9:1, v/v) and centrifuged at 10,000 rpm for 10 min. The water phase in the top layer was discarded and the bottom layer (containing the mixture of inflamed cells and phages) of the Eppendorf tube was frozen quickly in liquid nitrogen. Then, the bottom part of the Eppendorf tube was isolated by sterile scissors and the precipitates were resuspended with 100 μl phosphate-buffered saline (PBS) then co-incubated with 900 μl *Escherichia coli* ER2738 (log phase) at 37 °C for 5 min. 9 ml LB was added and fully mixed for the next use. 10 μl prepared mixture was used to detect the titer, and another 10 μl was diluted with LB for detection of titer. Titer = the number of phage clones in the culture dish × dilution fold × 10^2. The amount of phages contained in the sample = titer × 10 ml. Other prepared samples were incubated in a shaking bed for 4.5 h, then purified with PEG/NaCl and 10 μl purified sample was used to detect titer. Enrichment ratio = phage recovered/phage was used. The above procedures were repeated for 3 rounds when reaching the stable enrichment level. 50 μl purified sample was transferred to a 1.5 ml Eppendorf tube, then thoroughly suspended in 200 μl iodide buffer, added with 750 μl dehydrated alcohol and incubated at room temperature for 10 min. After that, the sample was centrifuged at 4 °C, 12,000 *g* for 10 min. The supernatant was discarded and the precipitates were washed with 600 μl 70% alcohol, then centrifuged at 4 °C, 12,000 *g* for 10 min. The supernatant was discarded. The Eppendorf tube was dried in a 60 °C drying oven in a short time then re-suspended in 20 μl TE (tris ethylene-diamine-tetra acetic acid, buffered solution) for DNA identification.

2.3. PCR and DNA identification of selected phages

10 μl purified phage sample was heated at 98 °C in metal bath for 10 min then centrifuged at 10,000 *g* for 3 min. Forward primer: 5′-CACCTCGAAAGCAAGCTGAT-3′. Reward primer: 5′-CAACGCCTGTAGCATTCCAC-3′. PCR products containing the inserted sequence were supposed to be 300 bp, while products without inserted sequence were 258 bp. 25 μl PCR system contains: 10 mM dNTPs 0.5 μl, Taq DNA polymerase 0.25 μl, 10 × PCR buffer (containing 15 mM $MgCl_2$) 2.5 μl, 10 μM forward and reward primer 1 μl, phage DNA 1 μl and sterilized water 18.75 μl. The samples were pre-denatured at 94 °C for 2 min, denatured at 94 °C for 30 s, annealed at 56 °C for 30 s and extended at 72 °C for 30 s. The reaction was repeated for 30 rounds. 5 μl PCR products were imbibed and identified via agarose gel electrophoresis for 30 min. Phage DNA and PCR products were then quantitated via ultraviolet spectrophotometry and 500 μl PCR products were used as DNA sequencing templates. DNA sequencing was commercially performed by The Beijing Genomics Institute.

2.4. Bioinformatic analysis

Non-redundancy analysis was performed via CD-HIT (Cluster Database at High Identity with Tolerance, http://bioinformatics.org/cd-hit/). Sequence identity cut-off was set as 1 [26]. All the non-redundant 7-mer peptides were then submitted to an online phage display peptide analysis platform (PhDPAP, http://phdpap.fimmu.com/). All the 7-mer peptides were transformed into 3-mer peptide by every 3 continuous amino-acid residues forward and backward through the background process of PhDPAP. The frequency of each tripeptide was calculated and the P value of Poisson distribution was computed. The tripeptides with P value under 2.5×10^{-5} and the 7-mer peptides carrying these tripeptides were clustered using the on-line alignment tool ClustalW (http://www.genome.jp/tools/clustalw/) and several characteristic motifs were yielded, including tetrapeptides, pentapeptides or hexapeptides. All these characteristic motifs were then submitted to on-line BLAST (the basic local alignment search tool, http://www.ncbi.nlm.nih.gov/blast) program for sequence similarity analysis, with the purpose of finding the target functional protein. The yielded functional proteins by BLAST were then submitted to SignalP 3.0 online service (http://www.cbs.dtu.dk/services/SignalP/) [27] to predict the secretory