Journal of Bioscience and Bioengineering
VOL. 122 No. 3, 287–293, 2016

www.elsevier.com/locate/jbiosc

Screening, expression, and characterization of an anti-human oxidized low-density lipoprotein single-chain variable fragment

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Received 19 November 2015; accepted 29 February 2016
Available online 30 March 2016

Increased levels of oxidized low-density lipoprotein (OxLDL) in the blood circulation are correlated with atherosclerosis. Monoclonal antibody-based detection systems have been reported for OxLDL. We identified novel single-chain variable fragments (scFvs) having affinity for human OxLDL and related ligands. We constructed an scFv library from nonimmunized human spleen mRNA. Two types (γ+κ and μ+λ) of scFv phage libraries were enriched by biopanning, and five scFv clones with affinity for OxLDL were identified. The γκ5 scFv, which showed the highest affinity for OxLDL, was cloned into pET-22b(+) and expressed in *Escherichia coli* BL21(DE3). γκ5, expressed as an inclusion body in BL21(DE3), was refolded and purified. The specificity and sensitivity of γκ5 were analyzed using enzyme-linked immunosorbent assays (ELISAs). The γκ5 scFv showed affinity for OxLDL and acetylated LDL. The sensitivity of γκ5 to low concentrations (1–2 μg/mL) of OxLDL was higher than that to AcLDL and LDL. Finally, we developed a sandwich ELISA using γκ5 and CTLD14 (a lectin-like OxLDL receptor-1 ligand recognition region), which allowed specific detection of OxLDL at a level below 0.1 μg/mL. Our results indicated that the γκ5 scFv was a promising molecule for the detection of modified LDL at very low concentrations.

[**Key words:** Single-chain variable fragment; Phage display library; Low-density lipoprotein; Oxidized low-density lipoprotein; Enzyme-linked immunosorbent assay; Protein refolding]

Low-density lipoprotein (LDL) is a carrier of cholesterol in blood. Increased levels of LDL in the blood promote the uptake of LDL into the arterial intima, where LDL is subjected to enzymatic and nonenzymatic modifications. Modified LDL is involved in the induction of atherosclerosis and other vascular diseases (1,2). In particular, oxidized LDL (OxLDL), which is present in atherosclerotic lesions of humans and animal models (3,4), may play a key role in the induction of atherosclerosis. Additionally, there is a correlation between increased blood OxLDL levels and the severity of acute coronary syndromes; therefore, the blood OxLDL level has been used as a biochemical risk marker for atherosclerosis and other vascular diseases (5,6).

OxLDL is not uniform, but rather occurs as complex structures. A number of detection systems for measuring OxLDL and other modified LDLs using monoclonal antibodies have been reported (6–10). These antibodies recognize one of the oxidation-specific epitopes of OxLDL, such as malondialdehyde (MDA) or oxidized phosphorylcholine (PC). Recently, a chicken monoclonal antibody for apolipoprotein B (ApoB) was reported (11). An assay system combining the monoclonal antibody and LOX-1, a receptor for OxLDL (12), was developed to measure the level of real potent risk for atherosclerosis.

During the last decade, recombinant single-chain variable fragment (scFv) antibodies (13) have been developed. ScFvs are good alternatives for antibodies raised from immunized animals because of their rapid and economic production, and they can be easily functionally modified through mutation. Additionally, scFv and Fab antibody fragments can be displayed on phage surfaces (14,15). A desirable scFv can be selected easily and rapidly from a repertoire of scFvs using phage display technology. In particular, nonimmunized scFv libraries can provide antibodies against a wide range of antigens and can be utilized for the selection of a desired antibody, even if an antigen cannot stimulate the immune system. Thus, scFvs are likely to be a powerful tool for constructing a detection system because of their specific molecular recognition ability and ease of handling. Recently, an anti-LDL(−) (electronegative LDL) scFv was cloned, and its ability to inhibit the uptake of LDL(−) by macrophages was demonstrated (16).

In this study, we constructed a nonimmunized human scFv phagemid library and selected scFvs that reacted with OxLDL. One selected scFv was expressed in *Escherichia coli*, refolded, purified, and analyzed for its ability to recognize OxLDL and related ligands. Finally, we constructed a novel, convenient ELISA for the detection of LDL-derived molecules such as OxLDL.

MATERIALS AND METHODS

Bacterial strains, vectors, and helper phage Phagemid pCANTAB 5E and M13KO7 helper phage were purchased from GE Healthcare (Buckinghamshire, UK).

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.02.014

E. coli amber suppressor strain TG1 [Δ(*lac-proAB*), *supE*, *thi*, *hsd*Δ5/F′(*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)] was purchased from Agilent Technologies (Santa Clara, CA, USA). Plasmid pET-22b(+) and *E. coli* strain BL21(DE3) [F$^-$, *ompT*, *hsdS*$_B$($r_B^- m_B^-$), *gal*, *dcm*, λ(DE3)] were purchased from Merck (Darmastadt, Germany). Plasmid pCR2.1TOPO and *E. coli* strain DH5α were purchased from Life Technologies (Carlsbad, CA, USA).

scFv gene library construction An scFv gene library was constructed according to the methods of Marks et al. (17) and Hawkins et al. (18) with modifications. First-strand cDNA was synthesized from human spleen poly A$^+$ RNA (BD Biosciences, Franklin Lakes, NJ, USA) using a first-strand cDNA synthesis kit (GE Healthcare) and primers specific for the constant regions of the γ and μ heavy chains and the κ and λ light chains (Supplementary Table S1A). Genes for the variable region of heavy chains ($V_{H\gamma}$ or $V_{H\mu}$) were amplified by polymerase chain reaction (PCR) using VH reverse and JH forward primers, and those for the light chains (V_κ or V_λ) were amplified using a set of V_κ back and J_κ forward primers or a set of V_λ back and J_λ forward primers (Supplementary Table S1B). The PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min followed by 25 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. Then, linker peptide $(Gly_4Ser)_3$ sequence was added to the V_H and V_L genes that were assembled into V_H–$(Gly_4Ser)_3$–V_L scFv genes as follows: V_H genes were amplified by PCR using VH back and JH forward linker primers, and V_L genes (V_κ or V_λ) were amplified by PCR using a set of V_κ back linker and J_κ forward primers or a set of V_λ back linker and J_λ forward primers (Supplementary Table S1B). Then, the V_H and V_L fragments with linker sequences were assembled by two-step PCR. Cycling conditions for the first PCR were as follows: 5 min at 94°C followed by seven cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. To the first PCR mixtures, the VH reverse primer and either J_K or J_λ forward primer were added. The second PCR was carried out for 25 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. Then, restriction sites (*Sfi*I and *Not*I) were added at the 5′ and 3′ ends of the scFv genes by PCR using VH back *Sfi*I and J_κ or J_λ forward *Not*I primers (Supplementary Table S1C). All PCR assays were carried out using *TaKaRa Ex Taq* HS (Takara, Kusatsu, Japan).

scFv phage library construction The scFv gene fragments were digested with *Sfi*I and *Not*I and ligated into the pCANTAB 5E phagemid vector. For expression, the phagemids were transformed into *E. coli* TG1 competent cells by electroporation. Transformed cells were counted to calculate the scFv library diversity. Transformed TG1 cells were infected with M13KO7 helper phage, and scFv-displaying phage particles were purified by PEG/NaCl precipitation. Purified phage particles were resuspended in 2× yeast extract and tryptone (YT; 0.5% NaCl, 1% yeast extract, 1.7% tryptone), mixed with an equal volume of 0.25% bovine serum albumin (BSA) containing 0.01% sodium azide and then used for biopanning.

Preparation of OxLDL Fully oxidized LDL (f-OxLDL) was prepared as described previously (19). Briefly, 1 mg/mL LDL (Biomedical Technologies Inc., Lancashire, UK) in phosphate-buffered saline (PBS) containing 5 μM $CuSO_4$ was incubated at 37°C for 20 h under dark and sterile conditions. Incubation was stopped by adding 1 mM EDTA, and the sample was dialyzed against PBS. Moderately oxidized LDL (m-OxLDL) was prepared using 2 mg/mL LDL containing 2 μM $CuSO_4$, followed by a 4-h incubation. The OxLDLs were filtered, and the protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). If necessary, 0.02% sodium azide was added to the prepared OxLDL.

Biopanning Biopanning was conducted in angled-neck cell-culture flasks (T-25; BD Falcon, Franklin Lakes, NJ, USA) using the f-OxLDL. An angled-neck flask was coated with 5 μg f-OxLDL by incubation at room temperature (RT) for 2 h. The flask was washed three times with PBS and blocked with 0.25% BSA at RT for 1 h. After the flask was washed three times with PBS, the YT solution of purified phage particles was added to the flask and incubated at 37°C for 2 h. After incubation, the flask was washed 40 times with PBS. Log-phase TG1 cells were added to the flask and cultured at 37°C for 1 h. Cultured TG1 cells were collected, spread on SOBAG (0.05% NaCl, 0.5% yeast extract, 2% tryptone, 2% glucose, 10 mM $MgCl_2$, 100 μg/mL ampicillin) plates and incubated at 30°C for 14 h. The TG1 cells were harvested from the plates and used for a second round of screening. The enriched phage clones were used to re-infect TG1 cells. The infected TG1 cells were cultured on SOBAG plates, and colonies were used separately for further screening by enzyme-linked immunosorbent assay (ELISA).

Selection of phage-displayed scFv using ELISA *E. coli* TG1 cells infected with enriched phage clones were separately precultured, inoculated at 10% in 2× YT-AG (100 μg/mL ampicillin, 2% glucose) with M13KO7 phage, and cultured for 2 h at 37°C. The cells were collected by centrifugation at 1500 ×*g* for 20 min, resuspended in 2× YT-AK (100 μg/mL ampicillin, 50 μg/mL kanamycin), and cultured overnight at 37°C. Then, each culture was centrifuged at 1500 ×*g* for 20 min, and the supernatant was collected. The supernatant was mixed with an equal volume of 0.25% BSA and incubated at RT for 15 min. Each phage clone solution was used for ELISA. A 96-well polystyrene microtiter plate (BD Falcon) was coated with 100 μL/well f-OxLDL (5 μg/mL PBS) at RT for 2 h. The plate was washed three times with PBS and blocked with blocking buffer (0.25% BSA/PBS; 200 μL/well) at RT for 2 h. After the plate was washed three times, 100 μL/well of the phage solution was added and incubated at RT for 2 h. The monoclonal antibody LDL1A-2 was used as a positive control for OxLDL binding. The plate was washed three times, and 200 μL/well of blocking buffer was added. After incubation at RT for 10 min, 100 μL/well anti-M13 monoclonal antibody-horseradish peroxidase (HRP) conjugate (GE Healthcare) diluted 1:5000 in blocking buffer was added and incubated at RT for 2 h. The plate was washed six times, and 100 μL/well of the HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added for color development. The reaction was stopped by adding 100 μL/well of 2 N HCl, and the absorbance at 450 nm was measured using a microplate reader.

Selection of soluble scFvs by ELISA Soluble scFvs from OxLDL-reacted phage clones were prepared for further selection by ELISA. Precultures of OxLDL-reacted clones were inoculated at 10% in 2× YT-AG and cultured at 30°C for 2 h. The cells were collected, resuspended in 2× YT-AI (100 μg/mL ampicillin, 1 mM IPTG), and cultured at 30°C for 12 h. The cultures were centrifuged at 1500 ×*g* for 20 min, and supernatants were collected as soluble scFvs. Soluble scFvs were mixed with blocking buffer (0.25% BSA/PBS) and incubated at RT for 10 min. A 96-well polystyrene microtiter plate was coated with f-OxLDL and blocked with 0.25% BSA/PBS as described above. After washing the plate, 100 μL/well of soluble scFvs was added and incubated at RT for 2 h. The plate was washed six times with PBS, and 100 μL/well of anti-E tag antibody (GE Healthcare) diluted 1:1000 in 0.125% BSA/PBS was added. After a 2-h incubation at RT, the plate was washed six times with PBS, and 200 μL/well of blocking buffer was added. After incubation at RT for 10 min, 100 μL/well of anti-mouse IgG (Fc-specific) peroxidase conjugate (GE Healthcare) diluted 1:60,000 in blocking buffer was added and incubated at RT for 1 h 30 min. The plate was washed six times with PBS, TMB was added for color development, and the reaction was stopped with HCl. The absorbance at 450 nm was measured using a microplate reader.

Expression of γκ5 scFv in *E. coli* using the pET system The γκ5 scFv gene was introduced into the pET-22b(+) expression vector to obtain a sufficient amount of the protein. *Nde*I and a *Xho*I restriction sites were added to the 5′ and 3′ ends of the γκ5 scFv gene, respectively, by PCR amplification using the γ+κ back and γ+κ forward primers (Supplementary Table S1D). The fragment was subcloned into the pCR2.1TOPO vector. The pCR2.1TOPO vector containing the γκ5 scFv gene was digested with *Nde*I and *Xho*I, and the γκ5 fragment was purified and ligated into the *Nde*I/*Xho*I site in pET-22b(+). The pET-22b(+) containing the γκ5 scFv gene was transformed into *E. coli* BL21(DE3) for expression. Transformed BL21(DE3) cells were cultured in LB medium (0.5% NaCl, 0.5% yeast extract, 1% tryptone, 50 μg/mL ampicillin) at 37°C with vigorous shaking until the culture reached an optical density at 600 nm (OD_{600}) of 0.5–0.6. Protein expression was induced with 1 mM IPTG for 4 h. The culture was centrifuged at 6000 ×*g* at 4°C for 10 min, the pellet was washed with PBS and resuspended in PBS containing protein inhibitor, and the cells were disrupted by sonication. The cell extract was centrifuged at 10,000 ×*g* for 10 min, and the pellet was collected to recover the γκ5 scFv inclusion bodies.

Refolding and purification of γκ5 Refolding of γκ5 scFv was performed according to the methods of Machida et al. (20). Fifty milligrams (wet weight) of inclusion bodies were resuspended in 150 μL PBS and unfolded by adding a mixture of 450 μL of 8 M guanidine hydrochloride (GdnHCl) with 6 μL of 4 M dithiothreitol (DTT) and incubating at 25°C for 1 h. Forty milliliters of 0.1% cetyltrimethylammonium bromide (CTAB) in PBS with 400 μL of 200 mM DL-cysteine was added to the unfolded protein solution, and the mixture was allowed to stand for 1 h at 25°C. Next, 10 mL of 3% cycloamylose (CA; Takara) solution, which acts as an artificial chaperone to encourage refolding, was added and incubated overnight at 25°C. The refolding solution was centrifuged at 16,000 ×*g* for 5 min, and the supernatant was collected as the refolded protein fraction. A 1/25 volume of 50% Ni-NTA agarose (Qiagen, Hilden, Germany) was added to the refolded protein solution and gently mixed at 4°C for 30 min. Protein-bound resin was collected by centrifugation at 1000 ×*g* for 5 min and washed twice with equal bed volumes of PBS. Protein elution was carried out in batch mode with 1 M imidazole in PBS. The eluted fraction was collected and dialyzed against a 400–600-fold volume of PBS. The dialyzed fraction was centrifuged at 20,000 ×*g* at 4°C for 30 min, and the supernatant was collected as purified soluble γκ5 protein. The protein concentration and purity were estimated by SDS-PAGE.

Direct ELISA for γκ5 scFv characterization A 96-well polystyrene microtiter plate was coated overnight with 100 μL/well of LDL (Biomedical Technologies Inc.), acetylated LDL (AcLDL; Biomedical Technologies Inc.), m-OxLDL, or f-OxLDL (0.05–10 μg/mL/PBS) at 4°C. The plate was washed three times with PBS and blocked with 200 μL/well blocking buffer (0.25% BSA/PBS) at RT for 2 h. After the plate was washed three times, 100 μL/well purified γκ5 (2.5 μg/mL PBS) was added and incubated at RT for 1 h. The plate was washed five times, and 100 μL/well anti-His tag HRP-conjugated antibody (R&D Systems, Minneapolis, MN, USA) diluted 1:1000 in blocking buffer was added. The plate was incubated at RT for 1 h and washed five times. TMB was added, and the reaction was stopped with HCl. The absorbance at 450 nm was measured using a microplate reader.

Sandwich ELISA for OxLDL detection A sandwich ELISA was developed using γκ5 and CTLD14, the LOX-1 ligand recognition region, as described previously (19). In order to determine whether the sandwich ELISA worked well to detect OxLDL in the range of human plasma, we compared LDL and f-OxLDL. Briefly, a 96-well polystyrene microtiter plate was coated overnight with 50 μL/well CTLD14 (20 μg/mL PBS) at 4°C, and the plate was washed three times with PBS.