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Development of a New High-throughput Screening Model for Human High Density Lipoprotein Receptor (CLA-1) Agonists[1]

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Objective To develop a new high-throughput screening model for human high-density lipoprotein (HDL) receptor (CD36 and LIMPII analogous-1, CLA-1) agonists using CLA-1-expressing insect cells. **Methods** With the total RNA of human hepatoma cells BEL-7402 as template, the complementary DNA (cDNA) of CLA-1 was amplified by reverse transcription-polymerase chain reaction (RT-PCR). Bac-to-Bac baculovirus expression system was used to express CLA-1 in insect cells. CLA-1 cDNA was cloned downstream of polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) into donor vector pFastBac1 and recombinant pFastBac1-CLA-1 was transformed into *E. coli* DH10Bac to transpose CLA-1 cDNA to bacmid DNA. Recombinant bacmid-CLA-1 was transfected into *Spodoptera frugiperda* Sf9 insect cells to produce recombinant baculovirus particles. Recombinant CLA-1 was expressed on the membrane of Sf9 cells infected with the recombinant baculoviruses. A series of parameters of DiI-lipoprotein binding assays of CLA-1-expressing Sf9 cells in 96-well plates were optimized. **Results** Western blot analysis and DiI-lipoprotein binding assays confirmed that CLA-1 expressed in insect cells had similar immunoreactivity and ligand binding activity as its native counterpart. A reliable and sensitive *in vitro* cell-based assay was established to assess the activity of CLA-1 and used to screen agonists from different sample libraries. **Conclusion** Human HDL receptor CLA-1 was successfully expressed in Sf9 insect cells and a novel high-throughput screening model for CLA-1 agonists was developed. Utilization of this model allows us to identify potent and selective CLA-1 agonists which might possibly be used as therapeutics for atherosclerosis.

Key words: HDL; SR-BI; CLA-1; Insect cells; High-throughput screening model; Agonist

INTRODUCTION

Atherosclerotic coronary heart disease (CHD) is one of the leading causes of death in modern society. Epidemiological, experimental, and genetic studies have demonstrated that high level of low-density lipoprotein cholesterol (LDL-C) and low level of high-density lipoprotein cholesterol (HDL-C) are associated with increased risk for atherogenesis[1-2]. Statins, HMG-CoA reductase inhibitors, are very effective in lowering plasma LDL-C and have become current mainstay for the treatment of heart disease. However, statins reduce cardiovascular events by only about 20%-40%[3]. Nonstatin therapies (either as monotherapy or in addition to statins) should be developed to be directed to novel therapeutic targets for the prevention and/or treatment of atherosclerosis. A growing body of evidence derived from clinical trials supports the contention that the raising of HDL levels may bring significant cardiovascular benefit independently of LDL lowering[4-5]. The next step in the management of atherosclerosis may center on HDL and the molecules with which they interact[6].

HDL delivers cholesterol ester to ovary, adrenal gland, and testis for hormone synthesis[7-8], and transports cholesterol from extrahepatic tissues to liver (called reverse cholesterol transport, RCT)[9-10]. In 1996, the scavenger receptor class B, type I (SR-BI) was reported as the first molecularly well-defined and functionally active cell-surface HDL receptor[11]. Human orthologue of SR-BI (hSR-BI) was initially identified as CD36 and LIMPII analogous-1 (CLA-1)[12]. The most well-known and studied hypothesis for the protective effect of HDL against atherosclerosis is related to its key function in

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RCT, i.e. SR-BI plays a key role in the HDL-C efflux and removal from the artery wall (the initial phase of RCT) and selectived HDL-C uptake in the liver (the last phase of RCT). SR-BI/apoE double knockout mice demonstrated dramatically accelerated onset of atherosclerosis and extensive cholesterol-rich coronary lesions[13]. In contradistinction, transient adenovirus-mediated hepatic overexpression of SR-BI in LDL-receptor-null mice fed with a Western-type diet leaded to regression of both early and advanced atherosclerotic lesions[14], and long-term transgenesis in heterozygous LDL-receptor-deficient mice fed with a high fat/cholesterol/bile-salt diet resulted in marked decreases in atherosclerosis[15]. These studies demonstrated the anti-atherogenic effect of HDL receptor SR-BI and SR-BI has been proposed to be a new therapeutic target for atherosclerosis[16-17]. The enhancement of SR-BI activity and/or its expression represents a potential strategy to promote significantly reverse cholesterol transport and this may inhibit the initiation and progression of atherosclerosis and promote its regression. Discovery and development of conventional pharmacological drugs that may increase SR-BI activity could be achieved through high-throughput screening by evaluating SR-BI-HDL interactions using *in vitro* cell systems.

To develop the high-throughput screening model for human HDL receptor CLA-1 agonists that may promote the activity of CLA-1 and accelerate reverse cholesterol transport, CLA-1 cDNA was cloned from human hepatoma BEL-7402 and expressed in insect Sf9 cells. Through a series of modified DiI-lipoprotein binding assays with recombinant CLA-1 on the plasma membrane of Sf9 cells, we have established the high-throughput screening model for CLA-1 agonists. Primary screening can define several positive samples from our compound library and microbial fermentation extract library.

MATERIALS AND METHODS

Cell Lines

Human hepatoma BEL-7402 cells were grown in RPMI-1640 medium (Gibco-BRL) containing 10% fetal bovine serum (FBS) at 37℃. Insect Sf9 (*Spodoptera frugiperda*) cells were grown and maintained in TNM-FH medium (PharMingen) supplemented with 10% heat-inactivated FBS at 27℃.

Cloning of cDNA Encoding Human CLA-1

Total RNA was extracted from BEL-7402 cells by SV total RNA isolation system (Promega). Advantage one-step RT-PCR kit (Clontech) was used to obtain the full-length human CLA-1 cDNA. Oligonucleotide primers were designed against human CLA-1 nucleotide sequence[12] in which the forward primer P1 (5′-AAAGATCTAGACATGGGCTGCTCCGC-3′) and the reverse primer P2 (5′-CCAGATCTGACCCTACAGTTTTGCTTCC-3′) contained *Bgl*II endonuclease restriction site (underlined). The resulting cDNA was subcloned into pGEM-T (Promega) and sequenced.

Construction of Recombinant Baculovirus

Recombinant baculovirus DNA was produced using Bac-to-Bac baculovirus expression systems according to the manufacturer's instructions. Briefly, human CLA-1 cDNA was subcloned into *Bam*HI site of pFastBac1. The recombinant plasmids were identified for the insertion orientation of the CLA-1 cDNA in pFastBac1 by restriction analysis with *Eco*RI (CLA-1 cDNA has an *Eco*RI site at 1050 bp and pFastBac1 has one at multiple cloning site). The resulting plasmid with the CLA-1 cDNA cloned in the same direction of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) was designated as pFastBac1-CLA-1.

pFastBac1-CLA-1 was transformed into *E. coli* DH10Bac containing bacmid (baculovirus shuttle vector plasmid) and helper plasmid (providing Tn7 transposition functions). The white colonies were selected for isolating the recombinant bacmid-CLA-1, resulting from the transposing of the mini-Tn7 element containing CLA-1 cDNA from the pFastBac1-CLA-1 to the mini-*att*Tn7 attachment site on the bacmid. The bacmid-CLA-1 was confirmed by PCR using a few pairs of primers including CLA-1 forward primer P1 and pUC/M13 reverse primer, CLA-1 forward and reverse primer P1 and P2, pUC/M13 forward primer and CLA-1 reverse primer P2, M13/pUC forward and reverse primer.

The minipreparations of recombinant bacmid-CLA-1 DNA were transfected into Sf9 cells using Cellfectin (Invitrogen). The recombinant baculoviruses were harvested at 72 hour post transfection.

Dot Blot

The labeled probe of CLA-1 cDNA was prepared using Gene Images random prime labeling module (Amersham). Sf9 cells were seeded at 5×10^4 cells/well in a 96-well plate in 100 μL TNM-FH supplemented with 10% heat-inactivated FBS, and followed by adding the recombinant baculoviruses at multiplicity of infection (MOI) of 5 for 72 hours. Sf9 cells were lysed in 500 mmol/L NaOH at room temperature, neutralized with 10 mol/L NH_4Ac and

transferred to nitrocellulose membrane by vacuum. After dryness at 80℃ for 2 hours, the nitrocellulose membrane was incubated in hybridization solution before adding the denatured probe and incubated overnight at 60℃. Following the stringent washes, the hybridization result was detected by Gene Images CDP-Star detection module (Amersham).

SDS-PAGE and Western Blot Analysis

Sf9 cells were grown in 6-well plates and infected with the recombinant baculoviruses at MOI of 5. At the indicated time points, pelleted cells were washed twice with cold PBS and then scraped and lysed in lysis buffer (50 mmol/L Tris, pH 6.8, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluroid, 50 μg/mL aprotinin, and 50 μg/mL leupeptin) at room temperature for 2 hours. Protein was separated on a 12% polyacrylamide gel and subsequently transferred to PVDF membrane. The PVDF membrane was blocked with blocking buffer (5% non-fat dried milk in Tris-buffered saline with 0.1% Tween 20) for 1 hour at room temperature. The rabbit anti-SR-BI/II antibody (Novus) was diluted in 1/5000 with blocking buffer and incubated with blocked PVDF membrane for 2 hours at room temperature. Then horseradish peroxidase-conjugated goat anti-rabbit IgG (1/30 000) was used for the detection with chemiluminescence reagent.

DiI-Lipoprotein Binding Assay

Sf9 cells were seeded at 5×10^4 cells/well in a 96-well plate in 100 μL TNM-FH supplemented with 10% heat-inactivated FBS, and followed by adding the recombinant baculoviruses at MOI of 5 for indicated time. Sf9 cells were washed twice by the Grace culture medium (Grace Medium 46.3 g/L). Binding of DiI-lipoprotein to Sf9 cells was carried out by incubating cells in 50 μL Grace culture medium containing 0.2% BSA with different concentrations of DiI-AcLDL (Calibiochem) and DiI-HDL (Biomedical Technologies) at room temperature for a few hours. After incubation, cells were washed twice with cold PBS, scraped by pipette and transferred to the black BMG plate in 100 μL PBS. Results were quantified with a PolarStar fluorescence plate reader at 540 nm excitation / 590 nm emission.

Data Analysis

The significance of the differences in fluorescence values between the sample group and the mock group was evaluated by Student's *t*-test. $P<0.05$ was considered statistically significant ($n=8$).

RESULTS

Construction of Recombinant Baculovirus Carrying CLA-1 cDNA

To express human high density lipoprotein (HDL) receptor CLA-1 on the plasma membrane of insect Sf9 cells, the Bac-to-Bac baculovirus expression system was used. Human CLA-1 cDNA was amplified by RT-PCR from human hepatoma BEL-7402 cells and cloned into pGEM-T vector. Sequencing results indicated that the sequence of the cloned cDNA was identical to that reported before[12]. The recombinant donor plasmid pFastBac1-CLA-1 was identified by restriction analysis with *Eco*RI (Fig. 1).

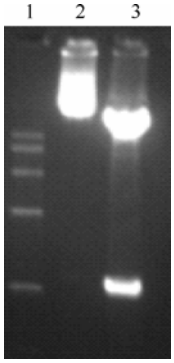

FIG. 1. Identification of pFastBac1-CLA-1 by restriction analysis with *Eco*RI. 1. DNA marker (2500 bp, 2000 bp, 1500 bp, 1000 bp, 500 bp); 2. pFastBac1/*Eco*RI; 3. pFastBac1-CLA-1/*Eco*RI.

The mini-Tn7 element containing CLA-1 cDNA on the pFastBac1-CLA-1 was transposed to the mini-*att*Tn7 target site on the bacmid. PCR was used to confirm that the human CLA-1 cDNA was transposed to the recombinant bacmid-CLA-1 (Fig. 2). The pUC/M13 amplification primers directed at sequences on either side of the mini-*att*Tn7 site were used independently (1.5 kb+2.3 kb) or in combination with the primers specific to human CLA-1 (P1 and P2). Amplification products from recombinant bacmid-CLA-1 are shown in Fig. 2.

Recombinant bacmid-CLA-1 DNA was transfected into insect Sf9 cells and the recombinant baculovirus was harvested and stored. Dot blot hybridization analysis confirmed that the human CLA-1 cDNA was transferred into the recombinant baculovirus (Fig. 3).

Expression of Human CLA-1 in Sf9 Cells

The Sf9 cells infected with the recombinant baculovirus showed a typical cytopathic effect 2 days after infection. At various time points after infection, cells were harvested and measured by Western blot

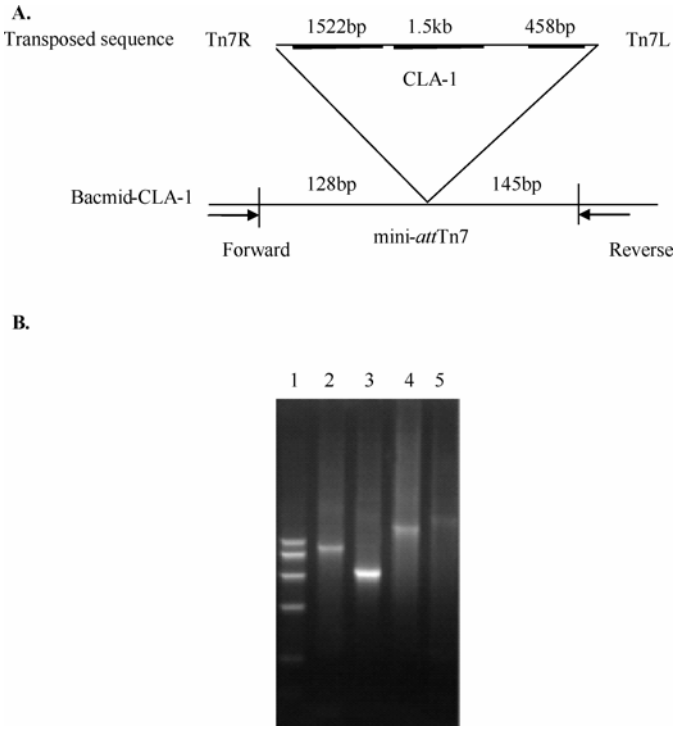

FIG. 2. Transposition region of recombinant bacmid-CLA-1(A) and identification of bacmid-CLA-1 by PCR (B). 1. DNA marker (2500 bp, 2000 bp, 1500 bp, 1000 bp, 500 bp). 2. PCR product using CLA-1 P1 primer and pUC/M13 reverse primer. 3. PCR product using CLA-1 P1 and P2 primer. 4. PCR product using pUC/M13 forward primer and CLA-1 P2 primer. 5. PCR product using pUC/M13 forward and reverse primer.

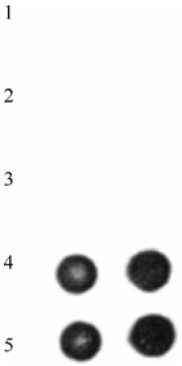

FIG. 3. Dot blot hybridization of Sf9 cells infected at MOI of 5 for 72 hours. 1. Sf9 cells; 2. Sf9 cells infected with AcNPV; 3. Sf9 cells infected with the wild-type baculoviruses generated by bacmid; 4. Sf9 cells infected with the recombinant baculoviruses generated by bacmid-CLA-1; 5. Bacmid-CLA-1 DNA.

analysis (Fig. 4). No immunoreactive protein was present in the cells infected with wild-type baculovirus. Human CLA-1 was detected as early as 24 hours post infection with recombinant baculovirus carrying CLA-1 cDNA, and the highest expression level of human CLA-1 was obtained at 96 hours post infection. Two major proteins of molecular masses 70 kDa and 85 kDa were detected by Western blot analysis.

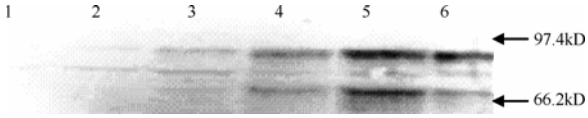

FIG. 4. Western blot analysis of human CLA-1 expressed in Sf9 cells. 1. Sf9 cells infected with wild-type baculovirus for 96 hours; 2-6. Sf9 cells infected with recombinant baculoviruses at 24, 48, 72, 96, and 120 hours respectively.

Ligand Binding Activity of CLA-1-expressing Sf9 Cells

Fluorescence-labeled human lipoproteins DiI-AcLDL and DiI-HDL were used to monitor the ligand binding activity of CLA-1 expressing on the plasma membrane of Sf9 cells. After the infection with recombinant baculovirus carrying CLA-1 cDNA (sample group) and wild-type baculovirus (mock group) for 72 hours respectively, Sf9 cells were incubated with DiI-lipoprotein for 6 hours before the measurement of bound fluorescence. Sf9 cells in the sample group might bind to DiI-AcLDL (Fig. 5) and DiI-HDL (Fig. 6) specifically compared with Sf9 cells in the mock group. The rate of ligand binding fluorescence values between the sample group cells and the mock group cells was elevated with the increasing concentration of DiI-AcLDL within the concentration detected. When the ligand DiI-AcLDL was at

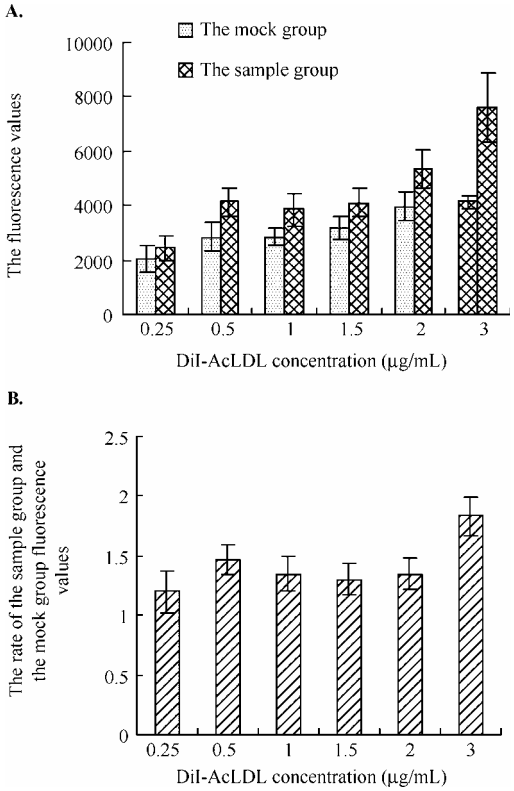

FIG. 5. Binding of DiI-AcLDL to CLA-1-expressing Sf9 cells at a MOI of 5 at 72 hours post infection.

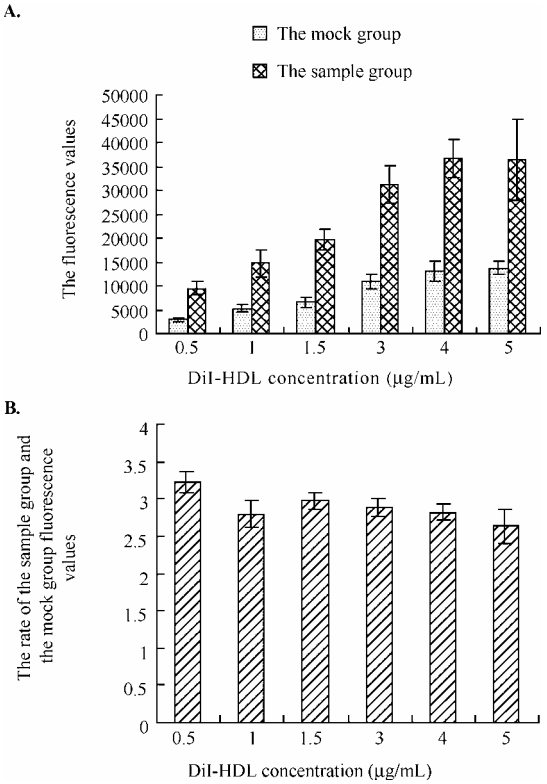

FIG. 6. Binding of DiI-HDL to CLA-1-expressing Sf9 cells at a MOI of 5 at 72 hours post infection.

the concentration of 3.0 μg/mL, the sample group bound to about twice ligand than the mock group. For DiI-HDL, the binding activity of increasing concentrations increased in a saturable fashion when DiI-HDL concentration was from 0.50 μg/mL to 5.0 μg/mL. The rate of ligand binding fluorescence values between the sample group cells and the mock group cells was around 3 and the highest rate was reached at a relatively low concentration of DiI-HDL (0.50 μg/mL). This result indicated that the human CLA-1 expressed by insect Sf9 cells was biologically active, for it could mediate the binding of CLA-1 ligands including the native lipoprotein HDL and the modified lipoprotein AcLDL.

Development of Screening Model for Human CLA-1 Agonists

For development of a high-throughput screening model for human CLA-1 agonists, the ligand binding assay based on the uptake of DiI-HDL by the recombinant baculovirus-infected Sf9 cells was further optimized. According to the conditions used above, the influence of the expression time of human CLA-1 on Sf9 cells and the incubation time of DiI-HDL on the sensitivity and efficiency of the assay were detected. Fig. 7 shows the effects of expression

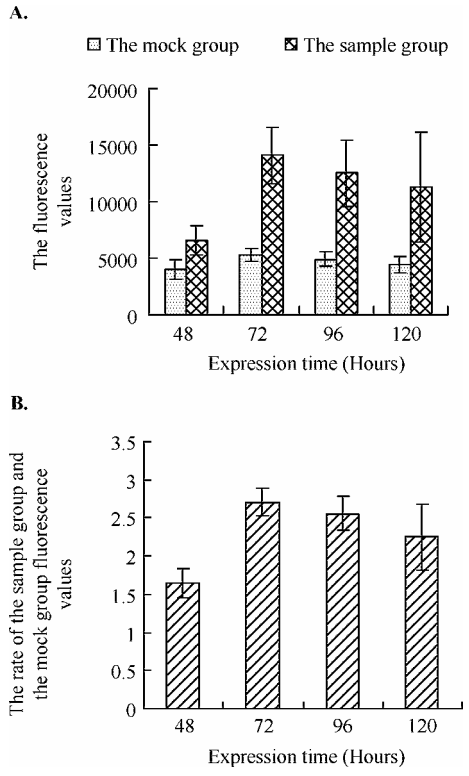

FIG. 7. Effects of expression time on assay at a MOI of 5 for 48, 72, 96, and 120 hours post infection respectively.

time on the assay. Both fluorescence values of the sample group and the rate between sample and mock groups were the highest at 72 hours post infection at 0.5 μg/mL DiI-HDL. Fig. 8 shows the effects of DiI-HDL incubation time on the assay. Although the fluorescence value increased with the increasing incubation time of DiI-HDL, the fluorescence rate between sample and mock groups was the highest when incubated with DiI-HDL for 6 hours. So the parameters used for the ligand binding assay was as follows: Sf9 cells were infected for 72 hours and then incubated with 0.5 μg/mL DiI-HDL for 6 hours.

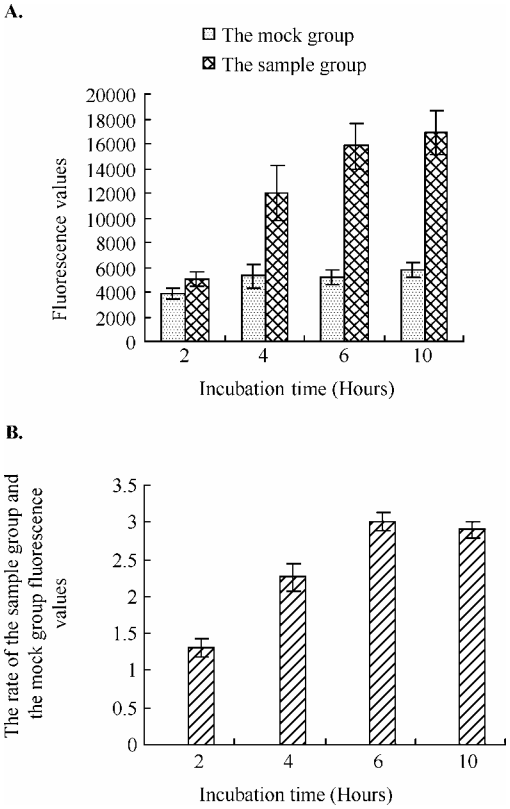

FIG. 8. Effects of DiI-HDL incubation time on assay at a MOI of 5 for 72 hours.

A high-throughput screening model for CLA-1 agonists was developed on the basis of the ligand binding assay. Screening of putative novel agonists was performed in the same 96-well plate, with recombinant baculovirus-infected Sf9 cells being incubated with DiI-HDL in the presence of test samples. A total of 4000 samples from our compound library and the microbial fermentation extract library were screened. One compound L34 (Fig. 9) and 3 microbial fermentation extracts (data not shown) showed concentration-dependent capability of increasing the DiI-HDL uptakes, suggesting that L34 might be a novel CLA-1 agonist.

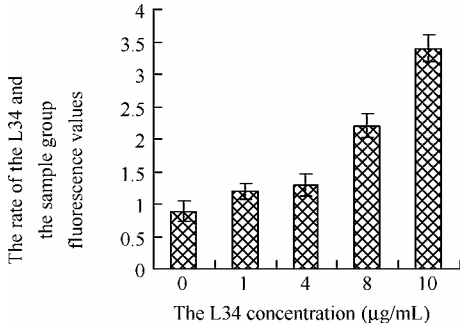

FIG. 9. Effects of L34 on DiI-HDL uptake in CLA-1 agonist screening model.

DISCUSSION

Decreased LDL after statin therapy has a major impact on cardiovascular morbidity and mortality. Now it is time to focus on HDL and its biological pathways as primary targets in the battle against atherosclerosis. Increasing HDL receptor activity in the liver or the artery wall can facilitate the flow of cholesterol and further reduce the morbidity of atherosclerotic CHD. For the development of pharmacological agents to modulate the activity of human HDL receptor, a high-throughput screening model was established using recombinant human HDL receptor expressed on the surface of insect cells.

Human HDL receptor CLA-1 is a 509-residue glycoprotein with a large extracellular loop anchored to the plasma membrane at both N- and C-termini by transmembrane domains which have short extensions into the cytoplasm[18]. The protein is heavily N-glycosylated, and palmitoylated on the cysteines in the C-terminal cytoplasmic and transmembrane domains. Because of the complication of post-translational modification and horseshoe-like membrane topologies of CLA-1, bioactive human CLA-1 could only be expressed in eukaryotic expression systems including mammalian cells[19], insect cells[19] and methylotroph yeast[20]. The yeast is a lower eukaryotic expression system and the cell wall is required to be removed for the ligand binding assay of recombinant receptors expressed on the plasma membrane. The expression of endogenous lipoprotein receptors in most cultured mammalian cell lines may impair the performance of ligand binding assay between the recombinant human CLA-1 and lipoproteins.

In most cases, recombinant proteins expressed in insect Sf9 cells using baculovirus expression system are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts. A

previous study[19] has demonstrated that human CLA-1 could be expressed on the plasma membrane of Sf9 cells with a high-affinity specific binding activity for the lipoproteins HDL, LDL, VLDL, OxLDL and AcLDL similar to that expressed on mammalian cells. Reaven *et al.*[21] discovered that microvillar channels can be induced by expression of recombinant rat SR-BI in Sf9 cells, and these specialized structures facilitate both the binding of HDL and selective HDL-CE uptake. Moreover, insect cells recognize a different set of lipoproteins other than mammalian cells, i. e. insect cells exhibit a negligible binding of human lipoproteins[19]. Thus, CLA-1 expressing insect Sf9 cells shows a high capacity and specificity of selective cholesterol uptake and may be developed and used as a novel screening model suitable for high-throughput screening.

In this study, biologically active recombinant human HDL receptor CLA-1 was expressed in Sf9 cells using Bac-to-Bac baculovirus expression system. This system allows rapid and efficient generation of recombinant baculovirus, greatly reduces the time it takes compared to conventional methods. Human CLA-1 expressed in C32 melanoma cells is a single band with an apparent molecular weight of 85 kDa by electrophoresis analysis[19]. In our study there were two major immunoreactive bands (around 70 kDa and 85 kDa) detected by Western blot analysis. This heterogeneity might result from the different degree of N-glycosylation by Sf9 cells.

The fluorescent compound is a useful tool in assessing activity of the scavenger receptors and screening for receptor antagonists[22]. To investigate whether recombinant CLA-1 expressed in Sf9 cells had the ability to bind to its lipoprotein ligands, DiI-AcLDL and HDL were selected to represent its scavenger receptor activity and HDL receptor activity respectively. The results showed that recombinant baculovirus carrying CLA-1 cDNA conferred to Sf9 cells, the capability to bind to both the modified lipoprotein AcLDL and native HDL. However, the ligand binding activity to HDL was much higher than that to AcLDL, especially at low concentration of DiI-lipoproteins (Fig. 5 and Fig. 6). This result is consistent with the saturation assays of different lipoproteins[19]. The possible reason is that HDL is a physiologically relevant authentic ligand of CLA-1. To get the high sensitivity of the assay and to mimic physiological situation of the receptor, the fluorescent ligand DiI-HDL was used for the development of the receptor agonist screening model.

Finally, after optimization of a series of conditions, the DiI-lipoprotein binding assay of recombinant CLA-1 expressed on insect cell plasma membrane was developed as a novel CLA-1 agonist screening model suitable to high-throughput screening. A pilot screening trial proved the feasibility of this established screening model and the possibility to find out small-molecule CLA-1 agonists. Utilization of this novel screening model will allow the identification of potent human HDL receptor CLA-1 agonists, which may facilitate reverse cholesterol transport to provide added benefit in the fight against atherosclerosis.

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