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Cell surface display of rabbit MCP1 on human embryonic kidney 293T cell line

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As atherosclerosis is a prevalent non-communicable disease, and yet, no definitive medical treatment found for it, Trapping MCP1 as a key factor in inflammation could be effective. Therefore, we decided to display rabbit MCP-1 (R-MCP1) on human embryonic kidney 293T cell line surface. Firstly, R-MCP1 plasmid (pR-MCP1) containing kappa chain signal sequence, R-MCP1 sequence and PDGFR intra membrane domain was constructed. The delivered pR-MCP1 was transformed in *E. coli* TOP10F*'*, and the resulted clones were assessed by PCR and digestion. After linearizing pR-MCP1 by *Bgl*II, HEK cells were transfected by them. MCP1 gene integration and expression was confirmed at RNA and protein levels by real- time PCR and flow cytometry, respectively. PCR product gel electrophoresis on genomic DNA of transfected HEK cells showed a 737 bp band. Based on real- time PCR results, We observed R-MCP1 gene expression significantly increased in transfected cells (272.26 ± 37.32) compare to untransfected HEK 293T cells (2.67 ± 0.12) ($p = 0.001$). The results of flow cytometry showed that about 85% of transfected cells were positive and express R-MCP1. Therefore, cell surface display of R-MCP1 has successfully been performed and the produced cells can be used in future research to prepare diagnostic and therapeutic agents like aptamers.

Keywords: Cell surface display, rabbit MCP-1, expression vector, gene expression, transfection, transformation

Introduction

Vascular diseases like atherosclerosis and restenosis are initiated and progressed by vessel wall inflammation[1] and accumulation of leukocytes in the arterial intima is considered as a key step in the inflammation process. *In vivo* and *in vitro* evidences support the pivotal role of monocyte chemoattractant protein 1 (MCP1) in regulating migration and infiltration of monocytes across the vessel walls[2-4]. In addition, MCP-1 stimulates vascular smooth muscle cell proliferation, angiogenesis, and oxidative stress which can initiate inflammation in sub-endothelium area[5].

The MCP1 is a member of C-C chemokine subfamily of chemokines which acts through C-C chemokine receptor 2 (CCR2) which is not observed in normal arteries. Endothelial cells, vascular smooth muscle cells and macrophages in atherosclerosis areas are source of MCP1 expression[6].

Several animal studies have revealed that knocking out the MCP1 or its corresponding receptor genes; reduce extent of atherosclerosis[7, 8].

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A short oligonucleotide sequence (RNA or DNA) named aptamer has been suggested as novel therapeutic agents and an appropriate substitution for monoclonal antibodies and specifically binds to corresponding ligands with high affinity[9,10]. Systematic Evolution of Ligand by Exponential Enrichment (SELEX) is a method for aptamer selection. One of the most frequent SELEX methods is cell SELEX which seems the cell type is the crucial in selecting appropriate aptamer in this process. A specific aptamer against MCP1 binds to MCP1 protein and could consider as a therapeutic agent to inhibit inflammation initiation and promotion. To select anti-MCP1 aptamer, target cells with overexpression of MCP1 on cell surface are needed. Thus, construction of cells which express the target protein is inevitable to make the aptamer selection process possible.

For MCP1 specific aptamer presentation as a new generation drug, several steps of clinical trials should be designed, on animal and human models. To reach this goal an aptamer against animal and human target protein must be selected. A special SELEX method which used to choose such aptamers is Toggle-SELEX[11].

In this process, two distinct cells which overexpressed the human and animal target protein are required. On the

other hand, rabbit is one of the useful large animal models for atherosclerosis[12]. Hence, we decided to produce a transformed HEK 293T cell which display rabbit MCP1 (R-MCP1) on its surface (HEk293T/R-MCP1).

Materials and Methods

Plasmid Construction

A 573 bp sequence contains *XhoI* and *NheI* restriction enzymes site in 3' and 5' ends, 76 bp of signal sequence of murine Ig kappa chain and HA tag, 317 bp of R-MCP1 cDNA between two *KpnI* enzyme sites and 150 bp of intramembrane domain of PDGFR was ordered to clone in PUC57R plasmid to GeneCust Company (Fig. 1A). The pcDNA 3.1 hygro + and the constructed cDNA was separately digested by *XhoI* and *NheI* enzymes (Thermo Scientific, USA) according to manufacturer's instruction. The digestion products (backbone of pcDNA 3.1 hygro + and cDNA of R-MCP-1) were electrophoresed and purified from agarose gel by DNA extraction kit (Bioneer). Linearized pcDNA 3.1 hygro + and R-MCP-1 were ligated by T4 DNA ligase enzyme (Thermo Scientific, USA). Ligation product was transformed into chemically competent *E.coli. TOP 10 F'*[13] and the resulted clones were analyzed by restriction digestion (with *XhoI* and *NheI*) and colony PCR, and named as pcDNA/R-MCP1. Colony PCR was done with forward (F) pcDNA primer 5'- ACTAGAGAACCCACTGC TTACTG-3' and reverse (R) pcDNA primer

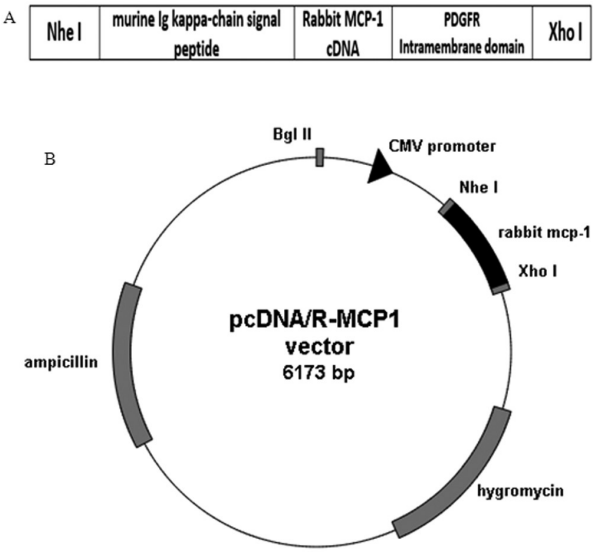

Fig. 1 — A: Synthesized cDNA containing rabbit MCP-1 B: Expression vector of pcDNA/R-MCP1

5'- ATGGCTGGCA- ACTAGAAGG-3' using a mix of *Taq* DNA polymerase (0.25 µl, 1.25U), 10X buffer (2.5 µl), 10 mM dNTPs (0.5 µl), 1.25 mM Mgc^{l2} (1 µl), ddW (17.75 µl) and 1 µl (10 mM) of each forward and reverse primers for pcDNA backbone. The PCR program was started with 1 cycle at 94°C for 4 min, continued by 30 cycles at 94°C for 30s (denaturing), 60°C for 30s (annealing) and 72°C for 1min (extension), and ended with 1 cycle at 72°C for 5 min in BioRad thermo cycler (Bio-Rad Laboratory, USA).

Amplification and Linearization of pcDNA/R-MCP1

E. coli TOP 10 F contains pcDNA/R-MCP1 was grown in Luria-Bertani (LB) (Sigma-Aldrich, St. Louis, MO, USA) broth medium (including 100 µg/ml ampicillin), in aerobic condition on 250 rpm shaker at 37ºC overnight. The plasmid was extracted by Sol Gent plasmid extraction kit (Korea).

The extracted pcDNA/R-MCP1 was linearized using *Bgl*II enzyme in 200 µl volume (20µl 10X buffer, 5µl enzyme and 175µl plasmid). The linear plasmid was cut from agarose gel and extracted by gel extraction kit.

Transfection

Cell Culture

Human embryonic kidney (HEK) 293T cells were obtained from Pasteure Institute of Iran. The cells were cultured in DMEM (Sigma-Aldrich) medium supplemented with 1% PS (Gibco) and 10% FBS (Sigme-Aldrich) 5% CO subscript at 37ºC.

In Vitro Transfection

Transfection was performed using TurboFect (Thermo Scientific) based on its protocol. Twenty four hours before transfection 1×10^6 HEK 293T cells were sub-cultured in two 25T culture flasks so that confluency of adherent cells on the time of transfection was 60-70%. Then, for each flask 7.5 µg of the linear pcDNA/R-MCP1 and pLOX/CWGFP plasmids were added to 750 µl serum free-DMEM medium separately, suspended by pipeting, and let to be equivalent for 1-2 min. About 15 µl of Turbo Fect reagent was added to the plasmid suspension and incubated for 15-20 min at room temperature. After that, the content of the tube was added to the cell culture flask drop wise, mixed them by swirling and incubated at 37ºC overnight. The day after, culture medium was changed, and from two days later, the cells were treated with 150 µg/ml hygromycin for

3 weeks. Transfection with pLOX/CWGFP was done to check the transfection efficiency. Re-transfection with the plasmid was performed on the selected cells in similar condition 3 weeks later.

Polymerase Chain Reaction (PCR) on HEK 293T Cell Genomic DNA

To confirm the integration of linear pcDNA/R-MCP1 into genome of HEK 293T cell, genomic DNA of the transfected and untransfected cells were extracted by Genet Bio DNA extraction kit (Genetbio-USA) according to manufacturer's instruction. PCR was done on extracted genomic DNA with forward and reverse pcDNA primers. The PCR process was performed as described above. Genomic DNA of untransfected HEK cell was used as the negative control. PCR products of the control and the positive samples were analyzed by 1% agarose gel electrophoresis comparing to 1 kb DNA ladder.

Relative Real-Time PCR

Total RNA of 10^6 transfected and untransfected cells were extracted using RNX kit (CINAGEN, Iran) based on the manufacturer's protocol. Using spectrophotometry and electrophoresis, quantity and quality of the extracted RNA were measured. The extracted RNAs were treated by DNase I (Thermo Scientific) according to its protocol and cleaned up by Bioneer DNA extraction kit. cDNA was synthesized from 1 µg of the total RNA by first strand cDNA synthesis kit (Thermo Scientific - USA). Relative real-time PCR was performed on the cDNA in a 20 µl volume using forward MCP-1 primer 5'- TACTGCTG CTCTGGGTTCC-3' and reverse MCP-1 primer 5'- TCACGGAGATGGTCTTGTTG-3' and forward beta actin primer 5'- TTCGAGCAAGA GATGGCCA-3' and reverse beta actin primer 5'- CACAGGAC TCCATGCCCAG-3' as internal control. The reactions were carried out by SYBER Green Master Mixes (Thermo Scientific, USA) with the following program: 95°C for 10 min, continued by 40 cycles at 95°C for 15s and 60°C for 1 min. For evaluating efficiency of cDNA synthesis and PCR amplification, beta-actin was used as internal control in the real- time PCR.

Data analyzing was done using $2^{-\Delta\Delta Ct}$ method, and $\Delta\Delta$ *C*t (threshold cycle) = (*C*t R-MCP1 gene, treated group - *C*t beta-actin, treated group) - (*C*t R-MCP1 gene, untreated group - *C*t beta-actin, untreated group). The change in R-MCP1 mRNA expression was calculated as $2^{-\Delta\Delta Ct}$. All the reactions were performed in triplicate.

Flow Cytometry

Transfected and untransfected cells were placed in two flow cytometry tubes (suspension of 1×10^6 cells / 200 µl medium in each tube). The cells of 1 tube were stained with 1 µl FITC conjugated monoclonal anti-HA antibody (abcam, ab1789), and another one with appropriate isotype antibody (abcam, ab 400107) as control sample. Two tubes incubated in dark place at 4°C for 45 min. After washing with phosphate buffer saline (Gibco), 200 µl PBS was added to each tube, mixed and detected using flow cytometry (BD bioscience, NJ, USA) by accumulating up to 10,000 events per tube. The flow cytometric data were analysed by Cell Quest software.

Statistical Analyses

Real- time PCR results were analyzed by SPSS (version 20) software. Mean ± SD was reported for the RQs obtained in real- time PCR and Mann-Withney nonparametric test used to compare the data.

Results

Transformation

The map of pcDNA/R-MCP1 has been shown in Fig. 1B. After transformation, gel agarose electrophoresis of PCR product of an individual clone demonstrated a 737 bp band (Fig. 2A). After digestion

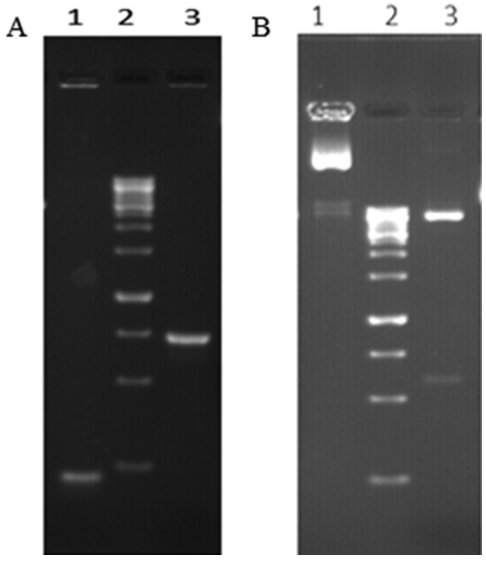

Fig. 2 — Confirmation of expression vector of pcDNA/R-MCP1 A: Gel electrophoresis of colony PCR product. Lane 1: a 224 bp band of colony PCR product on pCDNA/Hygro plasmid, lane 2: DNA ladder (1 Kb), lane 3: a 737 bp band of colony PCR product on pcDNA/R-MCP1; B: Gel electrophoresis of digestion with *Nhe* I and *Xho* I product. Lane 1: band of linearized pcDNA/Hygro plasmid, lane 2: DNA ladder (1 Kb), lane 3: a 573 bp band of fusion protein (intra-membrane domain of PDGFR and rabbit MCP-1) separated from the pcDNA/R-MCP1

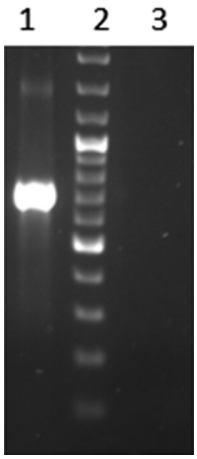

Fig. 3 — Gel electrophoresis of PCR product related to genomic DNA of transfected cells. Lane 1: a 737 bp band of integrated rabbit MCP-1, lane 2:DNA ladder (100 bp), lane 3: negative control

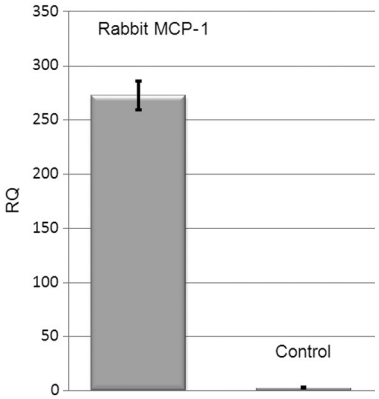

Fig. 4 — Rabbit MCP-1mRNA expression in transfected cells compare to untransfected ones based on real time PCR. column1: transfected cells with linearized expression vector of pcDNA/R-MCP1, column 2: untransfected cells

with *Xho*I and *Nhe*I, the CDS fragment (573 bp) was separated from the pcDNA/R-MCP1 (Fig. 2B). Also, sequencing findings proved the plasmid is containing fragment (results not shown).

Transfection

Compare to 100 bp DNA ladder, gel electrophoresis analysis of PCR products on genomic DNA has been shown in Fig. 3. Real- time PCR results revealed that compare to untransfected cells, R-MCP1 gene expression in transfected ones significantly increased (272.26 ± 37.32 versus 2.67 ± 0.12) ($p = 0.001$) (Fig. 4).

Flow cytometric analysis exhibited significant overexpression of R-MCP1 in transfected HEK 293T cells. So that 85% of the cells showed strong connection with the anti-HA tag antibody, while only 2% of the transfected cells stained by isotype antibody (Fig. 5).

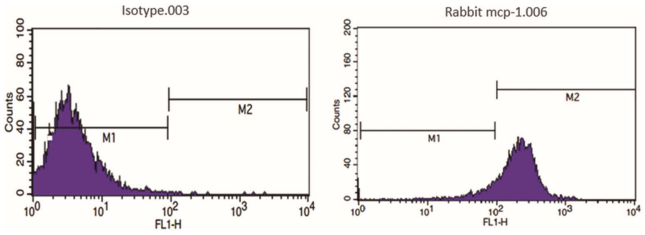

Fig. 5 — Expression level of the rabbit MCP-1 gene in HEK 293T cells; Flow cytometric analysis of Rabbit MCP-1 expression in: Isotypic control; and FITC conjugated Anti-HA tag antibody for rabbit MCP-1 overexpressing transfected cells

Discussion

As mentioned before, some therapeutic agents exert their function through trapping their protein targets. Despite of low yield, high cost and low efficiency of protein expression in eukaryotic systems, the native proteins are preferred to express in these systems because of accurate folding and proper post translational modifications especially when post translation modification is essential for protein function[14].

Many biological molecules are freely floating polypeptides in the cytoplasm, blood or in other body fluids. MCP1 is one of these molecules which is found in blood stream and injured endothelium as well as immune system cells[15]. Hence, to produce aptamer for such molecules by cell SELEX, cells with the protein of interest overexpression on their surface, in native state and natural folding and Post translation modification, are required. If the target protein is a membrane protein its native expression cassette is inserted into multiple cloning site (MCS) of pcDNA 3.1 Hygro+ but in the case of secretory or intracellular proteins some special plasmid generally named pDisplay should be used to display target protein on the cell surface[16].

Therefore murine Ig kappa-chain signal peptide and intramembrane domain of platelet derived growth factor receptor (PDGFR) of pDisplay vector were used in our expression vector for sorting and anchoring the protein to the cell surface, respectively[17]. Consequently, in this study we constructed eukaryotic cells for R-MCP1 expression to use in selecting anti R-MCP1 DNA aptamer and even other various therapeutic agents like antibody or nanobody.

Due to facile growth and convenience transfection of HEK-293T cells are usually used in cell biological researches[18]. In cell SELEX, cell with overexpression of target antigen on its surface is needed for positive selection and the same cell without expression needed

for counter selection. In the case of membrane proteins there is some positive cells in blood cells for example B lymphocyte express CD20 on their membrane and can be used for cell SELEX to select an aptamer against CD20 but there is not any CD20 minus B lymphocyte to use in counter SELEX. On the other hand, R-MCP1 is not expressed on 293T cells and 293T could be used for counter SELEX. To be confident of producing appropriate transformed cell, expression of R-MCP1 was evaluated at RNA and protein levels. PCR amplification on genomic DNA of the transformed cells was supportive for successful and stable plasmid integration into the genomic DNA of HEK cells. Real-time PCR results confirmed the expression of R-MCP-1 at RNA level.

Flow cytometry approach was used as a proper tool for detection the protein expression level on the transfected cell surface stained with FITC-conjugated anti-HA tag antibody. Regarding the significant staining obtained on the transformed cells, the R-MCP1 expressed on the cell surface with efficacy of more than 85%. The reason for applying anti-HA tag antibody to monitor the R-MCP1 extracellular expression was not finding any anti-R-MCP1 antibody. In addition peptide tags have various usages in biological researches like detecting and characterizing protein, protein trafficking, interactions and localization. Moreover, to distinct plasma membrane proteins from the cytoplasmic ones, tags are used in their extracellular domain and could be targeted by conjugated antibody to reveal protein expression on the membrane[19]. In conventional SELEX, the targeted antigen coats in chromatography column like HPLC column and for preparing the column we need a large amount of purified antigen and the expensive column and equipment but in cell SELEX selection of aptamer is feasible with cheap in house materials. Hence, in conclusion a cell with displaying the R-MCP1 on its surface has successfully been produced in this research. We believe that this transformed cell would help researchers for future biological and therapeutic agents development.

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