Construction and characterization of human embryonic kidney-(HEK)-293T cell overexpressing truncated α4 integrin

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Abstract

Blockade of α4 integrin by antibodies could be an appropriate treatment strategy in multiple sclerosis and Crohn’s disease. Considering disadvantages of antibodies, other elements (e.g. aptamers) have been proposed for antibodies replacement. Isolation of aptamers through cell-SELEX (systematic evolution of ligands by exponential enrichment) method requires positive and negative α4 integrin expressing cell lines. For a better isolation, we intended to construct a negative cell line lacking of specific ligand binding site of α4 integrin. Escherichia coli strain top 10 was used for truncated integrin subunit α4 (TITGA-4) expression. Human embryonic kidney (HEK)-293T cell was transfected with linearized TITGA-4 plasmid and subsequently screened for stable TITGA-4 expressing cells. Chromosomal DNA of TITGA-4-transfected cells was extracted and the presence of TITGA-4 gene in HEK-293T genome was confirmed by polymerase chain reaction (PCR). The expression level of TITGA-4 on HEK-293T cells was also analysed by real-time PCR and flow cytometry. Real-time PCR and flow cytometric analysis showed significant difference of TITGA-4 expression between untransfected HEK-293T cells compared to transfected cells. The results suggest that we have successfully constructed the truncated integrin α4 expressing HEK-293T cell, which will facilitate further research into the production of antibody, nanobody, and aptamer against α4 integrin.

Keywords: HEK-293T; Integrin α4; ITGA4; Multiple sclerosis.

INTRODUCTION

The pathogenesis of multiple sclerosis (MS) involves several steps including immigration of autoreactive immune cells to the central nervous system (CNS), inflammation, blood-brain barrier (BBB) breakdown and demyelination, though the mechanism of MS progression is not clearly understood. Since then numerous animal model studies confirmed and extended the predominant involvement of α4 integrin and vascular cell adhesion molecule-1 (VCAM-1) interaction in inflammatory cell recruitment into the CNS and pathogenesis of MS (1). Integrins are heterodimeric glycoprotein receptors that function as the major metazoan receptors for cell adhesion and connection of intracellular and extracellular environments (2,3).
Integrins exist as two non-covalently bound α and β subunits, which pair to form heterodimers (4,5). Integrins can bind to extracellular matrix (ECM) glycoproteins including collagens, fibronectins, laminins, and cellular receptors such as VCAM-1 and the intercellular cell adhesion molecule (ICAM) family (5,6). Integrin subunit alpha 4 (ITGA4) as a part of integrin α4β1 (CD49d/CD29) or very late antigen 4 (VLA-4) recognizes both type III connecting segment of fibronectin (3,7) and VCAM-1 expressed on activated endothelial cells and bone-marrow stromal cells (8-12). Previous studies indicated that the putative binding site for various ligands in ITGA4 structure is distinct but located in a small region of 107-268 residues. α4β1 is possibly involved in the interaction of leukocytes with activated endothelial cells which has a leading role in atherosclerosis, Crohn’s disease, and MS (13-16).

Regarding the α4 integrin role in MS pathogenesis, natalizumab (Tysabri®), a monoclonal antibody (mAb), was introduced to block α4 integrin and inhibit α4-mediated migration of leukocytes through BBB (17), that is still prescribed for relapsing-remitting MS (RRMS) patients (18). There are many disadvantages for mAbs including high immunogenicity and molecular weight and low heat stability, which led to vast researches on other agents to overcome the above problems. One of the selected agents for mAb substitution is aptamer (19) which is short single-stranded DNA or RNA sequences with low molecular weight and immunogenicity and high heat stability (20). Aptamers could bind to specific surface markers and inhibit their function, thus applied as diagnostic and therapeutic tools (21).

Systematic evolution of ligands by exponential enrichment (SELEX) process is used to select sequence of specific aptamers. Cell SELEX is the most convenient SELEX methods, in which protein targets expressed on the cell surface. Thus, cell types applied for negative and positive selection is of great importance in cell SELEX (20).

In this research, a recombinant human embryonic kidney-293 (HEK-293T) cell overexpressing truncated human ITGA4 (HEK-293/TITGA4) lacking ligand binding site was constructed. HEK293/TITGA4 could further be utilized as negative cell in SELEX process to isolate a specific aptamer against ITGA4 ligand binding site. The generated aptamer might be employed in future studies for treatment of MS and Chrohn's disease.

**MATERIALS AND METHODS**

**Transformation**

Z2827-M67-(ITGA4) expression vector (laboratory stock) encoding the human ITGA4, pcDNA3.1/Hygro (+) vector and *Escherichia coli* (E. coli) strain TOP 10F’ (Pasteur Institute of Iran, Tehran, I.R. Iran) as the host were used in this study. *E.coli* was grown in Luria-Bertani, (Sigma, USA) broth medium supplemented with 50 µg/mL tetracycline under aerobic condition and shaking at 250 rpm and 37 °C. *E.coli* was then separately transformed with the mentioned expression vectors in order to amplify the plasmid using CaCl2 chemical DNA transfer method. Subsequently, the transformed cells were spread on Luria-Bertani agar plates containing 100 µg/mL ampicillin incubated at 37 °C overnight. Appeared colonies were inoculated into Luria-Bertani broth containing ampicillin and incubated at 37 °C for 24 h with shaking at 250 rpm in order to extract the plasmid (Fermentas, Canada). The extracted Z2827-M67-(ITGA4) plasmids were digested with *Nhe*I enzyme (Thermo Scientific, USA) according to manufacturer's instruction to make a linear plasmid to apply for overlap polymerase chain reaction (PCR). The digested plasmids were then evaluated using agarose gel electrophoresis. Extracted pcDNA3.1/Hygro (+) was digested with *Bgl*II, treated with *Pfu* and finally digested with *Xho*I.

**Polymerase chain reaction**

Two 879 and 2312 bp segments of linear Z2827-M67-(ITGA4) were amplified using forward and reverse primers containing *Nhe*I and *Xho*I enzyme cutting sites, respectively. The PCR program for 879 bp segment amplification included a primary denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s,
72 °C for 2 min, with final extension step at 72 °C for 10 min. The PCR program for 2312 bp fragment amplification was exactly the same with an extension step of 72 °C for 4.5 min. The PCR products then were visualized by 1.5% agarose gel electrophoresis. The 879 and 2312 bp fragments were extracted from the gel using gel extraction kit (Bioneer, Korea). Overlap extension PCR was performed using a pair of overlapping primers (Table 1). The PCR program was initiated with one cycle at 94 °C for 4 min, continued by 5 cycles at 94 °C for 30 s and 58 °C for 7 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 7 min, and ended with a final extension cycle at 72 °C for 10 min. The overlap PCR product was then confirmed on 1% agarose gel. Overlap PCR product (3191 bp) was treated with XhoI (Fermentase, USA) restriction enzyme and finally subcloned in XhoI-treated Z2827-M67-(ITGA4) vector in a ligation reaction. The recombinant vector was named as pcDNA-TITGA4. Transformation of ligation product was then performed and positive clones were extracted and evaluated with XhoI and NheI. The extracted pcDNA-TITGA4 plasmids were linearized with NheI restriction enzyme.

Cell culture

The HEK-293T cell line (Pasteur Institute of Iran, Tehran, I.R. Iran) was grown in DMEM (Sigma, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 U/mL penicillin (Gibco, USA) and 100 µg streptomycin (Gibco, USA) at 37 °C in a 5% CO2 incubator.

Transfection of human embryonic kidney-293T cell

Human embryonic kidney -293T cells in exponential growth phase were seeded (5 × 10⁶ cells) a day before transfection. The medium was then changed with fresh medium 2 h before transfection. Transfection was performed using TurboFect reagent (Thermo Fisher Scientific, USA) based on manufacturer’s instruction. The transfected cells were grown in a non-selective medium for 48 h. Subsequently, the cells were exposed to 200 µg/mL hygromycin B (Gibco, USA) for 21 days. Hygromycin-resistant clones were randomly picked up, and cultured in separated flasks containing the medium supplemented with 200 µg/mL hygromycin B. The resistant clones are considered as stable transfected cell lines. The HEK-293T cell line which was left untreated, served as negative control.

DNA extraction and polymerase chain reaction on genomic DNA

Chromosomal DNA was extracted from TITGA-4-transfected cells using commercial extraction kit (BIONEER, Korea) according to manufacturer’s instructions. The presence of TITGA4 fragment in genome of transfected cells was assayed by PCR using specific primers designed for amplification of partial human TITGA4. The same program for amplification of fragment 879 bp was applied for verification of TITGA4 internalization into the HEK-293T cell genome. Agarose gel electrophoresis was performed to visualize the PCR product.

Table 1. The primer designation. Forward primer (PF) Nhel and reverse primer (PR) XhoI were used for amplification of 879 and 2312 bp fragments respectively. PF and PR over truncated α4 integrin (TITGA4) were used for overlap polymerase chain reaction (PCR). PF and PR TITGA4 were used for real-time PCR with the pair of β-actin primers as internal control.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>PF Nhel</td>
<td>5’ AAAGCTAGCTGCCACCTGACGCTCTAAAGA 3’</td>
</tr>
<tr>
<td>PR XhoI TITGA4</td>
<td>5’ CACTCGAGCTAATCATCATTGCTC 3’</td>
</tr>
<tr>
<td>PF Over TITGA4</td>
<td>5’ TGAGACCAGCCGAGCTGAGGAGCTCCCAAC 3’</td>
</tr>
<tr>
<td>PR Over TITGA4</td>
<td>5’ TCCTCCGACCAGCGGCTCTCAGTGC 3’</td>
</tr>
<tr>
<td>PF β-Actin</td>
<td>5’ ACCAACTGGAGGATATGGGAAAGA 3’</td>
</tr>
<tr>
<td>PR β-Actin</td>
<td>5’ TACGACCAGGAGCCATACAGGAGAAG 3’</td>
</tr>
<tr>
<td>PF TITGA4</td>
<td>5’ ACGTGCACAGACGCTCCAG 3’</td>
</tr>
<tr>
<td>PR TITGA4</td>
<td>5’ ACTCCATGCAAACCCACCCAG 3’</td>
</tr>
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Real-time polymerase chain reaction

Total RNA of about 10^6 transfected and control cells was extracted using RNX kit (Cinagen, I.R. Iran) and treated with DNase I enzyme (Thermo Scientific, USA). First strand cDNA was synthesized using 2 μg of the total RNA according to first strand cDNA synthesis kit instruction (Thermo Scientific). Relative quantification was performed by specific primers and β-Actin was used as internal control (Table 1). All real-time RT-PCR reactions were prepared in triplicate by mixing 10 μL of SYBR green master mix (Ampliqon kit, Denmark), 0.5 μL of each primer (10 μM), 2 μL of synthesized cDNA and 7μL RNase-free water. The reaction program was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Flow cytometric analysis

Human embryonic kidney (HEK)-293/TITGA4 recombinant cells were initially trypsinized, washed twice with phosphate-buffered saline (PBS) and then stained with anti-human CD49d antibody (Biolegend, USA) conjugated with phycoerythrin for 30 min at 4 °C. Cell suspension was then resuspended in 500 μL of PBS. Flow cytometric analysis was performed on FACS Calibur flow cytometer (BD Bioscience, USA) by accumulating up to 100,000 cells per tube. The untransfected cells were used as a negative control.

RESULTS

Truncated ITGA4 construction

Z2827-M67-(ITGA4) and pcDNA3.1Hygro (+) plasmids were applied in order to construct truncated ITGA4 through transfection, which were further verified subsequent to positive clone selection (data not shown). Subsequently, agarose gel electrophoresis results showed the amplification of two 879 and 2312 bp fragments of ITGA4 (Fig. 1a). Visualization of overlap PCR product also revealed a 3191 bp band (Fig. 1b).

Chromosomal DNA extraction and amplification

Genomic DNA of the transfected cells was extracted and PCR amplified. The PCR product illustrated amplification of an 879 bp DNA band on 1% agarose gel, which verified the integration of TITGA4 in to the HEK-293T cells (Fig. 2).

Fig. 1. Polymerase chain reaction (PCR) and overlap PCR results. (a) Amplification of two α4 integrin (ITGA4) gene fragments; lane 1, 879 bp fragment; lane 3, 2312 bp fragment; lane 4, marker 1kb. (b) Overlap PCR product; lane 1, marker mix, lane 2, 3191 bp fragment.

Fig. 2. Chromosomal DNA amplification. Agarose gel electrophoresis results illustrated the insertion of truncated α4 integrin (TITGA4) into the genome of transfected cells. Lane 1-3, amplified 879 bp fragment of transfected cells; lane 4, untransfected cell, and lane 5, marker 1 kb.
HEK-293T cell overexpressing truncated α4 integrin

Fig. 3. Real-time polymerase chain reaction (PCR) results. Truncated α4 integrin (TITGA4) expression level was significantly increased in transfected cells in comparison with negative control ($P < 0.001$) as illustrated in the graph.

Fig. 4. Flow cytometric analysis. The results showed the overexpression of α4 integrin (ITGA4) in transfected cells (a) when compared to untransfected cells (b).

Real-time polymerase chain reaction and flow cytometric analysis

The mean value of TITGA4 relative quantitation illustrated that expression level of TITGA4 was significantly increased in transfected cells (276 ± 2.9) compared with control cells (8 ± 0.8, $P < 0.001$) as shown in Fig. 3.

As shown in Fig. 4, HEK-293T cells have no expression of α4 integrin on their surface while the expression level of α4 integrin in HEK-293/TITGA4 cells was 76%, which correlates well with genomic DNA PCR amplification and real-time PCR results.

DISCUSSION

The role of α4 integrin in pathogenesis of a few diseases (15) has been approved and thus blockade of this molecule has been considered as a promising therapeutic approach. Aptamers have been introduced as appropriate candidates to achieve this purpose. Aptamers were developed by application of a SELEX method, cell SELEX, as the most common technique of aptamer preparation amongst different available strategies. Cell-SELEX requires both positive and negative cell lines to facilitate the aptamers development against the target of interest (20). In the current study we constructed an artificial HEK-293T cell enable to overexpress truncated α4 integrin to serve as a negative cell for SELEX. The HEK-293T cell line was transfected with TITGA4 lacking ligand binding site. The integration of TITGA4 in HEK-293T cells was evaluated using PCR on genomic DNA, which was followed by assessment of TITGA4 gene expression on
RNA and protein levels. Real-time PCR results revealed a great number of \textit{TITGA4} copy that was in accordance with flow cytometry data showing 76\% transfected cells overexpressing \textit{TITGA4}. A recent study constructed HEK-293T cells overexpressing \(\alpha_4\) integrin for further use as a positive cell in SELEX strategy (22). In this study, PCR and flow cytometry methods were used similarly in order to verify \(\alpha_4\) integrin integration into genomic DNA and the protein expression, respectively (22). In three separate studies, exactly the same procedures were applied to conduct the generation of recombinant cells overexpressing HBsAg (23), MCP-1 (24) and TIM-1 (25) for diagnostic and therapeutic purposes. Greater similarity of the target with intact molecule results in a more specific aptamer isolation. Moreover, limiting of the target to one or a few specific sites in target structure leads to reduction of cross-reaction and side effects of aptamer in future.

Mammalian cells including HEK-293T are widely used in laboratory research (26). Due to post-translational modifications (PTM) of proteins in eukaryotic cells like HEK-293T and production of appropriately folded protein, mammalian cells are considered as a good choice for target gene expression (25). There are several lines of evidences which have shown the use of same cell due to necessity of PTM or precise assembly of target. For instance Aricescu \textit{et al.} showed the importance of using mammalian cells like HEK-293 for expression of targets which have a high rate of PTM or need to be supra-assembled (26).

There are three general systems for transferring gene into the cells including biological, chemical and physical methods (27,28). In the present study, a chemical method with polycationic reagent was applied to transfer \textit{TITGA4} cDNA into the HEK-293T cells. Due to the possibility of viral DNA integration into the cell genome (29,30) biological method was not used in this study. Moreover, as physical methods need special instruments (29), unavailable in our laboratory, were not used in the current study. Transfection efficiency of 76\% using chemical methods indicates high efficiency of the method for transferring genes into the cells.

Recombinant HEK-293T cell overexpressing \textit{TITGA4} could further be applied in monoclonal and polyclonal antibody production, nanobody or aptamer selection against \textit{ITGA4} ligand binding site. This strategy leads to production of mentioned agents with high specificity and affinity against a particular site in target structure.

**CONCLUSIONS**

In the present study, we developed an artificial cell that overexpresses \textit{TITGA4}. This cell provides a platform for selection of aptamers with highest affinity and specificity against \textit{ITGA4} ligand binding site. This cell could be further considered as the negative cell to avoid inclusion of aptamers against all parts of \textit{ITGA4} structure in SELEX process.

**ACKNOWLEDGEMENT**

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