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Stable Expression of Recombinant RhD Antigen Isolated from Cord Blood in K562 Cell Line

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Abstract

Background: The Rh antigens are expressed as parts of a protein complex in the RBC membrane. This complex is a tetramer, consisting of two molecules of RhAG and two molecules of Rh proteins. To express RhD in RBC membrane, expression of RhAG is essential. This co-expression only occurs in the erythroid lineage. K562 cell line has an erythroid lineage.

Materials and Methods: Cord blood was used as a source of RHD gene in which nucleated RBCs are rich. Mononuclear cells were isolated using Ficol method. RNA was extracted by trizol followed by cDNA synthesis. RHD gene was isolated with specific primers. The RHD cDNA was ligated to pcDNA3.1 vector and cloned into E. coli. The recombinant pcDNA-RHD construct was transfected to K562 cell line. Stable cells expressing RhD were selected in the presence of geneticin. RT-PCR and western blot analysis were performed to detect recombinant RhD.

Results: Stable cells expressing recombinant RhD were established. RT-PCR results showed exogenous expression of recombinant RhD which was further confirmed by western blot analysis.

Conclusion: Overall, our results revealed that K562 is suitable for expression of RhD. The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein.

Keywords: rhd – k562 cell line, recombinant protein, stable cell

Introduction

Rhesus (Rh) is a highly complex blood group system in humans deeply rooted in transfusion medicine through implications in alloimmune transfusion reactions, hemolytic disease of the newborn, auto-immune hemolytic anemia, and the non-immune hemolytic condition associated with Rh-deficiency syndrome [1]. Of the Rh system antigens, antigen D is the most clinically significant because it is highly immunogenic. This high degree of immunogenicity stems from the fact that the entire RhD protein is absent from the erythrocyte membranes of persons expressing D-negative phenotypes.[2,3] The RhD and CcEe proteins arise from the RHD and RHCE genes, respectively, whereas the RhAG component arises from the RHAG gene.[4,5] Rh antigen expression occurs early in hematopoietic differentiation, being detected in colony-forming unit-erythroid (CFU-E) cells, but not the burst-forming unit-erythroid (BFU-E) cells.[1,6,7] It was hypothesized that cord blood might be a source for most hematopoitic progenitors including cells expressing RHD gene.[8,9,10] In this study, RHD gene was isolated from cord blood, cloned in bacteria and expressed in mammalian cells (eukaryotic expression system). The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein.

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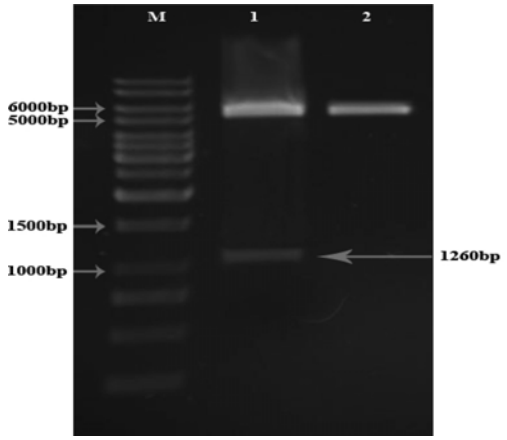


Figure 1: *Electrophoresis of digested plasmids on 1% agarose gel. The construct (lane1) and pcDNA3.1 (lane2) have been digested using EcoR 1 and Not 1. Lane1, pcDNA3.1-RHD; Expected size of insert, 1260 bp, can be observed in addition to plasmid while there is no similar band in the empty vector (Lane2). M; Molecular weight marker*

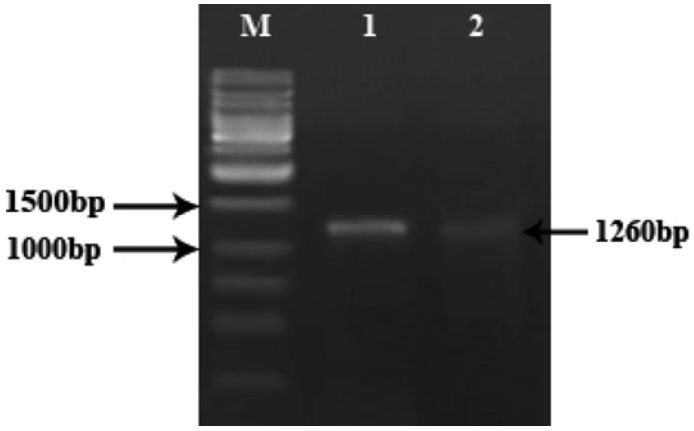


Figure 2: *To investigate whether the stable cells express RhD, RT-PCR was performed. Stable clones expressed more RhD mRNA (Lane1) compared with the cells transfected with empty pcDNA3.1 (Lane 2). M: Molecular weight marker.*

Materials and Methods

Plasmids and bacterial strains

Plasmid pcDNA3.1 (+) (Invitrogen, USA) was used for construction and expression. Bacterial strain was Top10 E. coli (Cinagen, Iran). The pcDNA3.1 (+) vector which provides the opportunity to clone the desired insert in bacteria and expresses in mammalian cell line was used in this study. It also contains neomycin resistance gene that allows selection of stable transfectants in the presence of geneticin in mammalian cells.

Cell culture

K562 (Erytroid leukemia cell line) was obtained from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. The cell lines were grown in RPMI-1640 medium (Gibco-BRL, Germany) with 10% fetal bovine serum (Gibco-BRL, Germany).

Isolation of mononuclear cells from cord blood

The Ficoll-Hypaque method was used to isolate mononuclear cells from the cord blood. Briefly, the blood was first diluted 1:1 with phosphate-buffered saline. Ten ml of the diluted blood was carefully layered onto a 3-ml Ficoll-Haque Plus cushion (Pharmacia Biotech, Uppsala, Sweden) in a 15-ml centrifuge tube (Falcon 3033 Becton Dickinson, Franklin Lakes, NJ). The tube was centrifuged at 400 xg for 30 minutes at 18–20°C. The interface (containing mononuclear cells) was carefully collected and washed twice with phosphate-buffered saline. The cells were used for RNA extraction.

RNA extraction

Total RNA from cell lines was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The quantity and quality of RNA was determined by spectrophotometry (Nanodrop, USA) and electrophoresis, respectively.

cDNA synthesis

Reverse transcription was performed by SuperScript III reverse transcriptase (Invitrogen, USA) with 1µg of total RNA followed by DNaseI (Invitrogen, USA) treatment and heat inactivation.

Amplification and cloning of RhD

The RhD cDNA was amplified using specific primers containing EcoR I and Not I sites. The sequence of forward primer was 5'-GC**GAATTC**ACCATGGTGAGCTCTAAGTACCCGCGGT CTGTCCGGC-3'.The sequences of reverse primer was, 5'-CTA**GCGGCCGC**TTAAAATCCAACAGCCAAATGAG-3'.

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All PCR reactions in aliquots of 50 µl contained 5 µl of 10X PCR buffer with MgSO4, 200 mM (each) deoxynucleoside triphosphates (dNTP), 15 pmol of each primer, and 200-500 ng of template DNA. PCR was carried out in Eppendorf Thermo Cycler using *Pfu* (Fermentas, Lithounia) with initial denaturation at 95°C for 5 minutes followed by 1 minute at 94°C, 45 seconds at 59°C and 45 seconds at 72°C for 30 cycles. PCR product was visualized by UV. The fragment of the expected size was cleaned up by High pure PCR purification kit (Roche Diagnostic, Germany) and digested with respective restriction enzymes. The fragment was ligated to pcDNA3.1 vector. The ligation product was transformed into competent Top10 E. coli and transformed cells were selected on Lauria Bertani plates containing 50 µg/ml ampicillin. The selected clones were further analyzed by Restriction enzymes and PCR and were finally sequenced by a commercial facility using universal forward, T7 promoter, and reverse BGH primers (TAG Copenhage, Denmark).

Plasmid construction and generation of stable RhD - expressing cell lines

The amplified RhD cDNA containing EcoRI and NotI restriction enzyme sites was cloned into the mammalian expression vector pcDNA3.1 (+) in the sense orientation. K562 cells were transfected with 2μg of pcDNA3.1–RhD using the FuGENE HD transfection reagent according to the manufacturer's protocol. (Roche, Germany). The pcDNA3.1 (+) DNA was used as a control. K562 cells stably expressing the pcDNA3.1-RhD construct were selected in a selective medium containing 800 μg/ml Geneticin (Roche, Germany) for at least 14 days. Several stable clones were generated by dilution of the cells and culture in 96-well culture plates. The expression of RhD was determined by RT-PCR and western blot analysis.

Detection of the expressed protein

Total protein was extracted by Complete Lysis B (Roche, Germany) according to manufacturer's protocol. Expressed protein was detected by running the samples heated in 1x SDS-PAGE sample buffer at 95° C for 5 minutes on 12% gel and stained with Coomassie blue. The proteins were also blotted onto PVDF paper (Hi-bond Amersham Biosciences, USA) and blocked with a solution containing 5% skimmed milk and 0.1% Tween 20. Blocked membranes were washed with PBS-0.05% Tween 20 and incubated with commercially available human anti-Rh(D) preparations (Santa cruz biotechnology, USA), a polyclonal antibody (with peroxidase conjugated goat anti-human IgG, IgM, IgA (Abcam, UK)), as the secondary antibody, at room temperature for 1.5 hours. Membranes were then washed 3 times with PBS containing 0.1 % Tween 20 and developed by DAB sollution (Sigma,USA).

Results

Isolation and Construction of RHD gene

Specific primers were designed to amplify full length of human RhD. The expected size of the PCR product was found to be 1260 bp. Then, the isolated fragment was cloned in pcDNA3.1 (+) vector and transformed to Top 10 E.coli. pcDNA3.1 (+) vector which was used for both cloning and expression. Existence of RhD in recombinant vector, pcDNA-RhD, was verified by restriction enzyme analysis (Fig1) and PCR and finally the accuracy of the nucleotide sequence of the gene and its frame in the vector was confirmed by DNA sequencing. The sequence of our gene is available in the Gene Bank with the access number of EU557240.

Expression of recombinant RhD in K562 cell line

To express RhD, K562 cell line was trasfected with pcDNA3.1- RhD. After culturing the cells in the presence of geneticin for two weeks, stable cell lines were generated. Then the stable cells were screened for RhD expression. To investigate whether the stable cells express RhD, RT-PCR was performed. Stable clones expressed more RhD mRNA compared with the cells transfected with pcDNA3.1 (Fig2) indicating the exogenous expression of RhD in K562 cell line. Next, western blot was performed to detect RhD protein. A single protein band in K562 cell lines tranfected with the construct (pcDNA3.1- RHD) was detected after developing but no expression was observed in the K562 cells transfected with pcDNA3.1 vector. This

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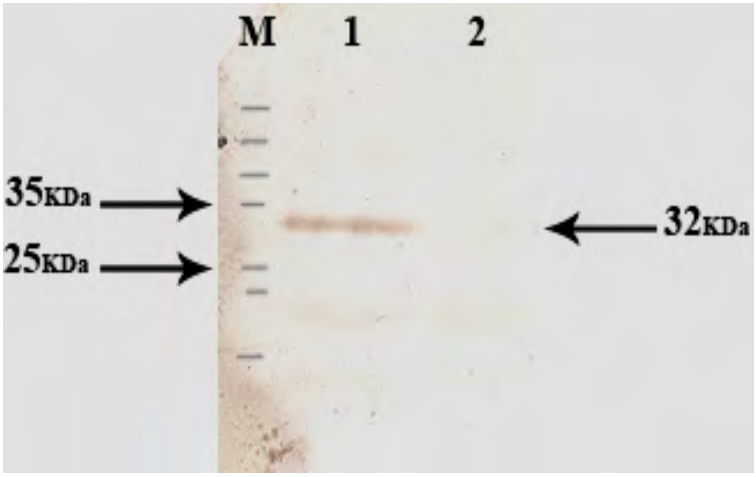


Figure 3: *Western blot was performed to detect RhD protein. A single protein band in K562 cell lines tranfected with the construct was detected after developing (Lane1) but no expression was observed in the K562 cells transfected with empty pcDNA3.1 vector (Lane2). M: Molecular weight marker.*

indicates that expression of RhD in K562 cell line (without construct) is too low to detect in protein level (Fig3).

Discussion

In this study, RhD from cord blood was isolated. The Rh blood group is one of the most complex blood groups known in humans.[1] Since its discovery 60 years ago when it was named (as a mistake) after the Rhesus monkey, it has become the second important blood group in the field of transfusion medicine.[11,12] It has remained of primary importance in obstetrics, being the main cause of hemolytic disease of the newborn (HDN). Since RBCs lack nucleus, it would be impossible to obtain mRNA of RhD from RBCs. Progenitors of RBCs in peripheral blood expressing RhD gene are too low to obtain RhD mRNA while in cord blood, they are adequately available.[1,6,7] In this study, cDNA of RhD was isolated from cord blood. DNA sequencing showed the accuracy of the RhD gene. This indicates that cord blood cells may represent a source for obtaining RhD gene. In this study, pcDNA3.1 expression vector was used for cloning and expression. The pcDNA3.1 is a chattel vector and contains human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells, and multiple cloning sites in the forward (+) and reverse (-) orientations to facilitate cloning. It also contains neomycin resistance gene for selection of stable cell lines in the presence of geneticin.[13-15] Recombinant RhD protein was detected as a single bound in western blot analysis indicating exogenous expression of RhD in k562. It is a notion that expression of RhD in protein level is too low to be detected by western blot in k562 cell line. However, Suyama et al. showed expression of RhD protein using immunopercipitation methods.[16]

In this study, FuGENE HD transfection reagent was used for transfection. FuGENE HD transfection reagent is a multi-component reagent that forms a complex with DNA, then transports the complex into animal or insect cells and provides high transfection efficiency in many common cell types. This reagent is suitable for both transient and stable transfection and here, for the first time, stable expression of RhD in K562 cell line is reported using FuGENE HD transfection reagent.

The role of RhAG in the surface expression of RhD was explored by Mouro-Chanteloup et al.[7] Nonerythroid HEK293 cells which lack Rh and RhAG were transfected with RhD and RhAG cDNAs using cytomegalovirus (CMV) promoter–based expression vectors. In HEK293 cells, a low but significant expression of RhD was obtained only when RhAG was expressed at a high level. Suyama

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et al. also showed similar results. However, they used nonerythroid COS-1 cell line instead of proerythroblast-like K562 cells for the expression of both RhAG and RhD.[17]

Expression of RhD has been reported in the K562 cell line by Yan et al.[18] They used different vectors for cloning and also different reagents for transfection in comparison to our study. The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein and could be applicable for the production of anti-D antibody in an animal model such as guinea pig.

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