Advanced Pharmaceutical Bulletin

Adv Pharm Bull, 2015, 5(1), 69-75
doi: 10.5681/apb.2015.009
http://apb.tbzmed.ac.ir

Research Article

Preparation of Proper Immunogen by Cloning and Stable Expression of cDNA coding for Human Hematopoietic Stem Cell Marker CD34 in NIH-3T3 Mouse Fibroblast Cell Line

Farzaneh Shafaghat[1,2,3], Hajar Abbasi-Kenarsari[4], Jafar Majidi[1,4], Ali Akbar Movassaghpour[5], Dariush Shanehbandi[1], Tohid Kazemi[6]*

[1] *Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[2] *Department of Immunology, International Branch of Aras, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3] *Students' Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.*
[4] *Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.*
[5] *Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[6] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*

Article info

Article History:
Received: 5 August 2014
Revised: 2 September 2014
Accepted: 9 September 2014
ePublished: 5 March 2015

Keywords:
· CD34
· Cloning
· Eukaryotic Expression
· HSCs
· KG1a

Abstract

Purpose: Transmembrane CD34 glycoprotein is the most important marker for identification, isolation and enumeration of hematopoietic stem cells (HSCs). We aimed in this study to clone the cDNA coding for human CD34 from KG1a cell line and stably express in mouse fibroblast cell line NIH-3T3. Such artificial cell line could be useful as proper immunogen for production of mouse monoclonal antibodies.

Methods: CD34 cDNA was cloned from KG1a cell line after total RNA extraction and cDNA synthesis. Pfu DNA polymerase-amplified specific band was ligated to pGEMT-easy TA-cloning vector and sub-cloned in pCMV6-Neo expression vector. After transfection of NIH-3T3 cells using 3 µg of recombinant construct and 6 µl of JetPEI transfection reagent, stable expression was obtained by selection of cells by G418 antibiotic and confirmed by surface flow cytometry.

Results: 1158 bp specific band was aligned completely to reference sequence in NCBI database corresponding to long isoform of human CD34. Transient and stable expression of human CD34 on transfected NIH-3T3 mouse fibroblast cells was achieved (25% and 95%, respectively) as shown by flow cytometry.

Conclusion: Cloning and stable expression of human CD34 cDNA was successfully performed and validated by standard flow cytometric analysis. Due to murine origin of NIH-3T3 cell line, CD34-expressing NIH-3T3 cells could be useful as immunogen in production of diagnostic monoclonal antibodies against human CD34. This approach could bypass the need for purification of recombinant proteins produced in eukaryotic expression systems.

Introduction

CD34 gene, located on long arm of chromosome 1, consists of nine exons and codes for single-chain type I transmembrane glycoprotein with molecular weight 115-120 KDa.[1,2] cDNA coding for human CD34 was first cloned and characterized by Simmons et al.[3,4] CD34 has two alternatively spliced full (long) and truncated (short) isoforms differ in cytoplasmic tail.[5,6] CD34 molecule is expressed on hematopoietic stem cells (HSCs) and progenitors, and also on high endothelial venules (HEVs) of lymph nodes.[7-10] This molecule belongs to sialomucin family and because of high glycosylation and several N- and O-linked sialylatedglycans, plays an important role in adhesion of hematopoietic cells to bone marrow stroma and in binding of L-selectin on naive T lymphocytes to HEVs, process plays pivotal role in homing of these cells to parafollicular region of lymph nodes.[1,11,12] Today, CD34 is specific selection marker for HSCs, primitive and rare cell population in bone marrow with ability to produce and differentiate to all blood cells including immune cells.[13-15] This marker has been widely used for identification, enumeration and isolation of HSCs in clinical and also research areas.[16,17] For these purposes, specific monoclonal antibodies (MAbs) against CD34 molecules are employed, and production of such useful tools are inevitable for better and more specific recognition of surface CD34.[1,18,19]

Production of MAbs by hybridoma technology was first introduced by George Kohler and Cesar Milestain.[20] Up to now, huge number of investigators have employed hybridoma technology, but with some modifications including different strategies for immunization of mice. Of them, some groups have stably expressed the gene

***Corresponding author:** Tohid Kazemi, Tel/Fax: +98 41 33364665, Email: kazemit@tbzmed.ac.ir

coding for protein of interest in mouse fibroblast cell line, NIH-3T3,[21] and have used the cells as immunogen.[22-24] Because of murine origin of NIH-3T3 cell line, the only immunogen part of stably transfected cells is ectopically expressed protein. Using this strategy, all problems encountered in purification of recombinant proteins in eukaryotic systems are bypassed, and intact protein with complete conformational structure is used as immunogen. In addition, transfection of cDNA coding for a specific protein in NIH-3T3 cell line has been performed for purposes other than immunization of mice, e.g. the signaling potential or functional properties of the molecule.[25-28]

Here, we reported cloning of long isoform of human CD34 cDNA and stable ectopic expression on mouse fibroblast cell line NIH-3T3 for further experiments to produce anti-CD34 monoclonal antibodies useful in diagnosis and even therapeutic approaches.

Materials and Methods

Cells and bacteria

KG1a and NIH-3T3 cell lines were purchased from National Cell Bank of Iran (NCBI, Tehran, Iran) and cultivated in RPMI 1640 cell culture medium (Gibco, Darmstadt, Germany) supplemented by 20% Fetal Bovine Serum (FBS) (Gibco, Darmstadt, Germany), 100 µg/ml Penicillin and 100 IU/ml Streptomycin (Gibco, Darmstadt, Germany) under humidified and 5% CO2 conditions. E.Coli strain DH5α was purchased from Promega Inc. (WI, USA) and cultured in Luria Bertani medium.

Flow cytometry

Evaluation of surface expression of CD34 molecule on KG1a, as a source for cloning of human CD34, was performed by indirect staining of KG1a cells. 5×10^5 cells were harvested and washed by PBS 1× containing 0.1% NaN3. Mouse monoclonal anti-human CD34 antibody (Biolegend, London, UK) was added on cells in final concentration of 5 µg/ml. In parallel, cells were stained with isotype control antibody (Biolegend, London, UK), as negative control. After 1 hour incubation at 4°C, cells were washed two times and then FITC-conjugated sheep anti-mouse immunoglobulin (Avicenna Research Institute, Tehran, Iran) was added in 1/50 dilution. Cells were incubated in a dark place for 1 hour at 4°C and after two times washing, they were scanned in flow cytometer (BD FACSCalibur flow cytometer).

Total RNA extraction and cDNA synthesis

5×10^6 KG1a cells were harvested and washed two times by RPMI 1640 culture medium. After final centrifugation, supernatant was completely discarded and the pellet was thoroughly resuspended. Cells were lysed by 1 ml RNX-plus solution (CinnaGen, Tehran, Iran) and total RNA was extracted according to manufacturer's recommendations. Briefly, after adding 200 µl chloroform (Merck, Darmstadt, Germany) and incubation for 5 minutes on ice, the solution was centrifuged at 12000 rpm for 15 minutes at 4°C and colorless aqueous phase was transferred to other tube carefully. Isopropanol (Merck, Darmstadt, Germany) in equal volume was added and after mixing and incubation for 15 minutes on ice, the solution was centrifuged and the precipitated RNA was seen. RNA was washed in 75% ethanol (Merck, Darmstadt, Germany) and resolved in RNase-free double distilled water. Quantity and quality of RNA was evaluated by UV spectrophotometry and agarose gel electrophoresis, respectively.

Five µg RNA was employed for synthesis of first strand cDNA using MMuLV reverse transcriptase (RT) enzyme (Thermo Fisher Scientific, Inc., MA, USA) and random hexamer (N6) primer (Thermo Fisher Scientific, Inc., MA, USA). Amplification of beta actin as a house keeping gene in polymerase chain reaction (PCR), as discussed later, was performed for confirming synthesis of cDNA.

Primer design and Polymerase chain reaction

To amplify human CD34 cDNA, specific primers were designed so that forward and reverse primers contained KpnI and HindIII restriction sites, respectively. Selection of restriction enzymes was on the basis of multiple cloning sites (MCS) of expression vector will used for expression of protein in eukaryotic system. For this purpose, reference sequence for human CD34 mRNA isoform 1 (NM_001025109.1) was obtained and the sequence was imported in NEBCutter web-based software (version 2.0) to find if the sequence has restriction sites for KpnI and HindIII restriction enzymes. The sequence of forward and reverse primers were selected from the beginning and ending part of reference sequence, respectively and were analyzed in OligoCalc web-based software to check for self-complementarity and hairpin formation of primers. Also, melting temperature (Tm) of both primers was calculated for upcoming experiments and adjusted against each other. To facilitate efficient translation in eukaryotic expression system during subsequent experiments, Kozak consensus sequence (GCCACC) was considered after the sequence for KpnI restriction site (at the beginning of forward primer) and upstream of the start codon. As presence of G nucleotide at position +4 is considered as strong consensus,[29] i.e. it maximizes the expression level of gene of interest, nucleotide +4 was replace by G nucleotide. To terminate the translation, on the other hand, stop codon was considered at the end of sequence for CD34 cDNA and before the sequence for HindIII restriction site. Optimized PCR conditions were obtained by performing reactions in different concentrations of $MgCl_2$ and a spectrum of annealing temperatures. Twenty-five µl reaction mixture contained 2.5 µl 10× PCR buffer, 1.5 µl 10 mM dNTPs (Thermo Fisher Scientific, Inc., MA, USA), 1µl each primer (10 pmol/µl), 0.2 µl Taq DNA polymerase (10 U/µl) (CinnaGene, Tehran, Iran) and 1 µl cDNA. Each PCR reaction was underwent initial denaturation at 95°C for 5 minutes followed by 39 cycles of denaturation (95°C for

30 seconds), annealing (different temperatures for 30 seconds) and extension (72°C for 45 seconds), and final extension at 72°C for 10 minutes. For obtaining PCR product with high fidelity, amplification of CD34 cDNA was performed using Pfu DNA polymerase (Thermo Fisher Scientific, Inc., MA, USA) in previously optimized PCR conditions, with the exception of 2.2 minutes for extension step, and A-tailing was done at 72 °C for 7 minutes using Taq DNA polymerase. PCR products were subjected to agarose gel electrophoresis, visualized by ethidium bromide at final concentration 5 µg/ml and documented in UVP Gel Documentation System (UVP, CA, USA).

TA-cloning of CD34 cDNA

After agarose gel electrophoresis, specific band with correct size was extracted using GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Inc., MA, USA) and then was ligated into pGEMT-easy vector (Thermo Fisher Scientific, Inc., MA, USA) using 3 units T4 DNA lygase (Thermo Fisher Scientific, Inc., MA, USA). After overnight incubation at 4°C, ligation mixture was transformed by heat shock method into DH5α competent bacteria, previously prepared by 0.1M $CaCl_2$ solution. After refreshing bacteria by adding fresh LB broth and incubation for 1 hour at 37°C and shaking in 150 rpm, they were transferred on LB agar medium containing 100 µg/ml Ampicillin (Dana, Tabriz, Iran), 40 µl X-Gal (20 µg/ml) (Thermo Fisher Scientific, Inc., MA, USA) and 40 µl IPTG (0.1mM) (Thermo Fisher Scientific, Inc., MA, USA). Plate was incubated overnight in 37°C and white colonies were evaluated by colony-PCR in conditions similar to conditions employed for amplification of CD34 cDNA. One positive colony was selected according to specific band with correct size and cultured in LB broth medium overnight at 37 °C and shaking in 250 rpm. Miniprep preparation was performed by Gene JET Plasmid miniprep kit (Thermo Fisher Scientific, Inc., MA, USA) according to manufacturer's recommendations. For initial confirmation of presence of a gene insert with correct size and proper restriction sites i.e. for KpnI and HindIII restriction enzymes (Thermo Fisher Scientific, Inc., MA, USA), double digestion was performed using 10 units of each enzyme overnight at 37°C. Digestion product along with undigested construct was run in agarose gel to visualize excised band. Consequently, selected construct was subjected for sequencing by T7 promoter and SP6 universal primers, to confirm accurate nucleotide sequence of insert. Chromatogram was analyzed first for good peaks for all nucleotides, and then entire sequence was aligned in NCBI database for checking proper sequence of inserted gene. On the other hand, flanking region of inserted gene was checked for vector-specific sequences to insure proper cloning vector and avoid cross-contamination by other commonly used vectors.

Preparation of pCMV6-Neo/CD34 recombinant construct

Double digested CD34 cDNA was sub-cloned in pCMV6-Neo digested in the same way. Ligation was done as previously described and competent DH5α bacteria were transformed by ligation mixture. Screening of colonies was performed on LB-Agar ampicillin plate and positive colonies were identified by colony-PCR reaction. Miniprep for one positive colony was prepared and direct sequencing using of V1.5 and XL39 primers was done for confirmation. To obtain high quality and quantity of finalized recombinant construct without any contamination by bacterial endotoxin, Maxiprep was prepared by EndoFree Plasmid Maxi Purification Kit (Thermo Fisher Scientific, Inc., MA, USA) according to manufacturer's recommendations.

Eukaryotic expression of human CD34 in mouse fibroblast cell line

NIH-3T3 mouse fibroblastic cells were cultured in the same conditions for KG1-a cells except of RPMI culture medium supplemented by 10% FBS. Cells were subjected to titration for resistance against G418. 10^5 cells were seed in 6-well cell culture plate and cultured in the presence of different concentrations (300 to 800 µg/ml) of G418 (Thermo Fisher Scientific, Inc., MA, USA). They were monitored for one week and minimum concentration of G418 for selection of NIH-3T3 cells was determined. 10^5 NIH-3T3 cells were cultured overnight in 6-well cell culture plate and transfected by the mixture of 3 µg pCMV6-Neo/CD34 recombinant construct and 6 µl JetPEI transfection reagent (Polyplus-transfection Inc. NY, USA) according to manufacturer's recommendations. In parallel, cells were transfected by expression vector devoid of CD34 cDNA (mock transfection). 48 hours after transfection, transient expression of CD34 antigen was examined in flow cytometry methods, as described earlier. To obtain stable expression, cells were cultured in complete cell culture medium containing G418. Starting concentration was determined by above-mentioned experiment on NIH-3T3 cells. Growing of transfected cells and dying of untransfected cells were monitored and live cells were subjected to gradually increasing concentrations of G418 up to 1500 µg/ml during two months.

Results

Surface Expression of CD34

Flow cytometric analysis of surface expression of human CD34 on KG1a myeloid cell line, as a source for amplification of CD34 cDNA, showed strong fluorescent detected in 99 % of cells by flow cytometer as M2. In contrast, surface staining of cells by isotype control, as negative control, showed very weak signal (M1: 1%).

Amplifying CD34 cDNA

Total RNA was purified from KG1a cells and cDNA was synthesized using 5 µg of total RNA. Processes were confirmed by agarose gel electrophoresis and beta actin gene amplification, respectively. Designed forward and reverse primers were 5´- GGTACCGCCACCATGGTG

GTCCGCAGGGGCGC-3´ and 5´- AAGCTTTCACAAT TCGGTATCAGCCACCACG-3´. They showed no self-complementarity and hairpin formation. On the other hand, the reference sequence had no restriction sites for selected restriction enzymes i.e. KpnI and HindIII. Using Taq DNA polymerase, conditions for amplification of CD34 cDNA were optimized. In 1 mM of MgCl2 and annealing temperature of 62°C, specific band with 1176 bp length (1158 bp for CD34 cDNA and 18 bp for two restriction sites and Kozak consensus sequence) was obtained and the reaction was repeated using Pfu DNA polymerase, but using 2.2 minutes extension time at 72°C. After A-tailing by Taq DNA polymerase, PCR product was run in agarose gel electrophoresis (Figure 1A) and specific band was then extracted.

TA-cloning of CD34 cDNA

Extracted PCR product and pGEMT-easy TA-cloning vector were mixed in ratio 5:1 and incubated overnight. Transformation of competent bacteria was performed using heat shock method and after refreshing of bacteria, they spread on LB agar containing ampicillin, X-gal and IPTG. After overnight incubation in 37°C, 8 colonies were obtained. Of them, 5 colonies were white and selected for colony-PCR reaction. Using the same PCR conditions for amplifying CD34 cDNA, 2 colonies showed strong single bands comparable to the size for CD34 cDNA (Figure 1B). One of the colonies was cultured overnight in LB broth medium containing Ampicillin. Preparation of Miniprep was performed, and quality and quantity were measured by agarose gel electrophoresis and UV spectrophotometry, respectively. According to cloning strategy, the construct was subjected to double digestion by KpnI and HindIII restriction endonucleases and successful excision of inserted CD34 cDNA was seen (Figure 1C). Initial analysis of two-sided sequencing of the construct showed good quality peaks and alignment of the inserted sequence in NCBI data base showed complete matching of cloned CD34 cDNA to reference sequence for CD34 mRNA isoform 1 (Figure 2).

Construction of recombinant pCMV6-Neo/CD34 construction

pGEMT-easy/CD34 recombinant cloning vector was digested by KpnI and HindIII restriction enzymes. Excised CD34 cDNA with 1176 bp band was ligated into double digested pCMV6-Neo expression vector by the same enzymes and pCMV6-Neo/CD34 construction was transformed into competent DH5α bacteria. After transformation, 8 colonies were obtained from cultured DH5α bacteria in LB agar/ampicillin medium. After performing colony-PCR reaction, 3 colonies showed successful insertion and one of the colonies was selected for preparation of Miniprep, direct sequencing and preparation of Maxiprep for subsequent experiments.

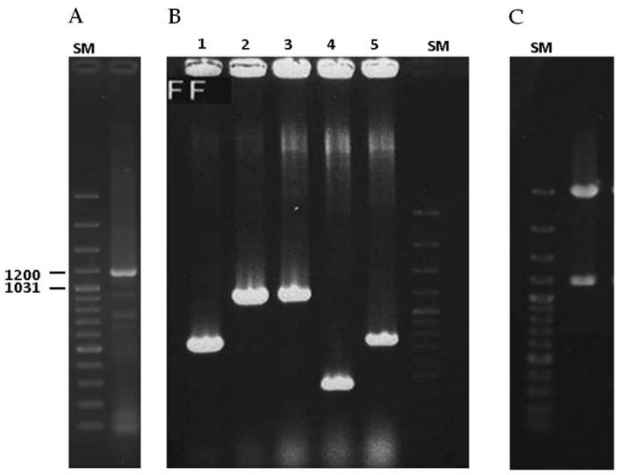

Figure 1. Cloning and subcloning of CD34 cDNA. (A) Amplification of specific band for human CD34 cDNA using Pfu DNA polymerase, (B) Colony–PCR reaction on five white colonies (1-5) after blue/white selection. (C) Excision of 1176 bp band for human CD34 cDNA after double digestion of the construct using KpnI and HindIII restriction enzymes. SM: DNA size marker (bp)

Transfection of NIH-3T3 cells and expression of human CD34 protein

NIH-3T3 mouse fibroblastic cell line was transfected by pCMV6-Neo/CD34 recombinant expression vector using JetPEI transfection reagent and transient expression of CD34 was confirmed by surface flow cytometry 48 hours after transfection (Figure 3D). After that, pCMV6-Neo/CD34 and mock-transfected and also untransfected NIH-3T3 cells were subjected to growth in complete cell culture medium containing 400 µg/ml G418, minimal dose for dying NIH-3T3 cells. Untransfected cells died and transfected cells were continued to be grown in G418 up to 1500 µg/ml during two months. Cells were finally analyzed by surface flow cytometry and significant positive reactivity of pCMV6-Neo/CD34-transfected cells (95%) (Figure 3E) was seen. On the other hand, untransfected and mock-transfected NIH-3T3 cells showed no positive signal in flow cytometry (3% and 4%, respectively) (Figure 3 B and C).

Discussion

CD34 molecule has long been used as a specific marker for hematopoietic stem cells (HSCs) in research and clinic.[30] For this purpose, polyclonal and monoclonal antibodies (MAbs) have been valuable tools, and several study groups tried to produce specific monoclonal antibodies against human CD34.[31] According to modified protocol introduced in 1970s for producing mouse monoclonal antibodies,[20] the first and one of the most important steps of hybridoma technology is preparing a good and proper immunogen. It should be as intact as possible for preserving all potential epitopes. On the other hand, using full-length protein with proper post transcriptional modifications similar to that occurs for native protein, is helpful for obtaining functionally more active and useful MAbs.[32] Additionally, immunization of animal with purified

protein is done by mixing antigen to complete (CFA) and incomplete Freund's adjuvant (IFA) usually administered subcutaneously. According to American Association for Laboratory Animal Science (IACUC) Policy on Administering Complete Freund's Adjuvant,[33] this protocol could induce unwanted local inflammation causing skin ulcerations and draining sinuses with granulomas and may lead to improper antibody response to antigen and limited number of antigen-specific clones. All above mentioned reasons force us to produce recombinant proteins in eukaryotic expression systems, in which purification step is tedious and challenging. However, immunization of mouse using stably-transfected murine NIH-3T3 cell line needs neither adjuvant nor purification and the route of injection is intraperitoneal with minimal side-effects for animal. Stable expression of cDNA coding for protein of interest in mouse fibroblast cell line, NIH-3T3, has been used by several investigators as an approach to prepare immunogen for immunization of mouse [22-24] and also for other research purposes.[25-28]

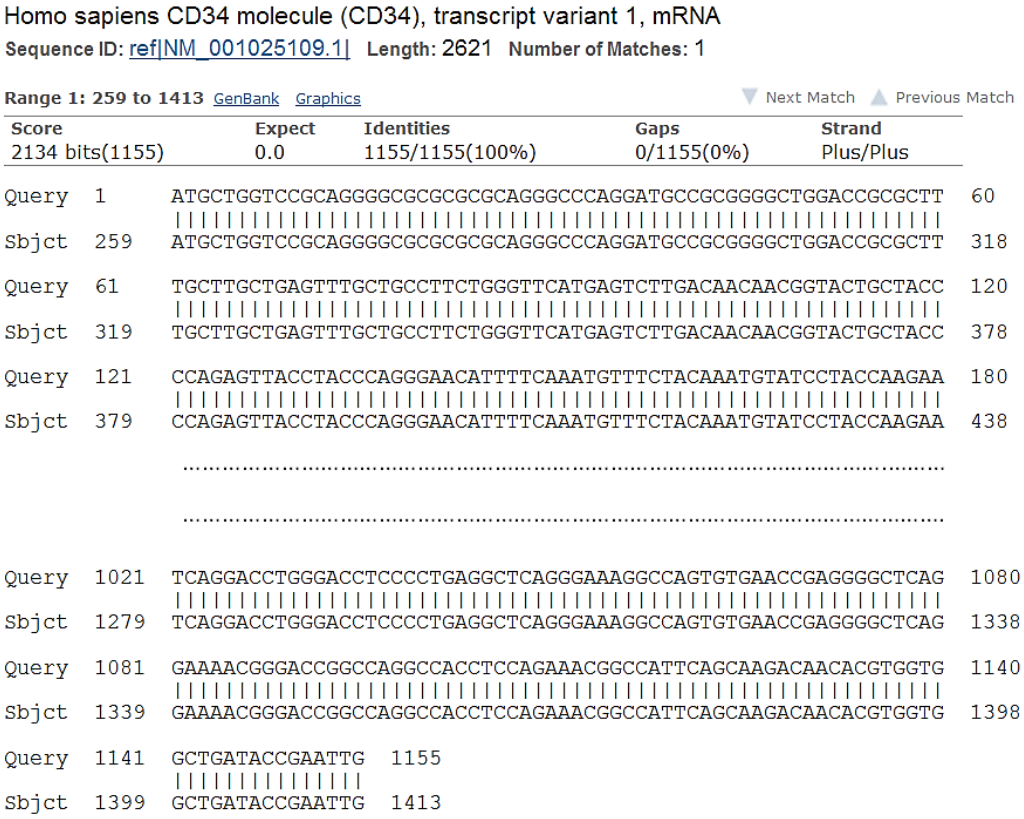

Figure 2. Alignment of amplified cDNA for long isoform of human CD34 to reference sequence in NCBI database. Comparing the 1158 bp amplified sequence with reference sequence for long isoform (variant 1) of human CD34 showed complete alignment. Briefly, 5' and 3' ends of alignment have been showed

KG1 is a myeloblastic cell line with $CD7^{-}$ $CD34^{+}$ phenotype and has been obtained from an old AML patient. This cell line was first identified and introduced in 1978. After 35 repetitive passages, a sub-lineage named KG1a ($CD7^{+}$ $CD34^{+}$) was obtained with absence of some characteristics of its own parent cell line, including response to colony-stimulating factor (CSF) and expression of FCγ receptor.[34] To insure high expression of CD34 on KG1a cells, they were subjected to indirect immunofluorescent staining, and results showed strong surface expression of CD34. So, KG1a was used in this study as a cellular source for expression of human CD34 gene and amplifying cDNA coding for CD34 protein.

Amplification of CD34 cDNA resulted in 1176 bp amplicon, subsequently cloned in pGEMT-easy TA-cloning vector and completely aligned to reference sequence of mRNA for long isoform of human CD34 isoform 1. cDNA was then sub-cloned in a eukaryotic expression vector for further transfection in NIH-3T3, as it has been reported to be a mouse fibroblast cell line.[35] For this purpose, pCMV6-Neo as an expression vector with a strong promoter for cytomegalovirus (CMV) was utilized for. pCMV6-Neo contains a resistance gene to G418 antibiotic (neomycin) and it is useable for screening of transfected cells. So transfected NIH-3T3 cells would be resistant and alive against G418 antibiotic.

Conclusion

In this study the cDNA coding for long isoform (transcript variant 1) of human CD34 was amplified and

cloned from KG1a myeloid cell line and stably expressed in mouse NIH-3T3 cell line. Stable expression of CD34 molecule in NIH-3T3 resulted in an appropriate immunogen for production of monoclonal antibodies useful in diagnostic and research areas.

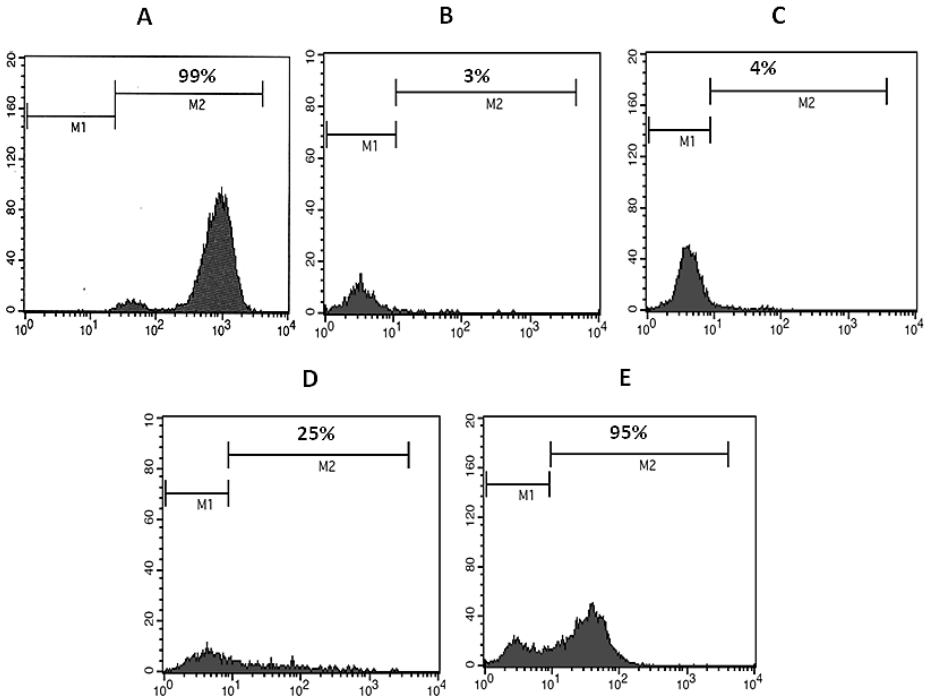

Figure 3. Flow cytometric analysis of expression of human CD34. Using specific monoclonal antibody, expression of human CD34 on KG1a (A), NIH-3T3 (B), mock-transfected NIH-3T3 (C) and pCMV6-Neo/CD34-transfected NIH-3T3 cells (D, for transient and E, for stable expression) were analyzed.

Acknowledgments

We would like to give special thanks to Leila Mohammad-nejad for her technical assistance. This study was supported by grant number 91.26 from Drug Applied Research Center, Tabriz University of Medical Sciences.

Ethical Issues

Not applicable.

Conflict of Interest

There is no conflict of interest to be reported.

References

1. Drenou B. Flow cytometry for CD34 determination in hematopoietic grafts. *Hematol Cell Ther* 1996;38(6):505-12.
2. Greaves MF, Brown J, Molgaard HV, Spurr NK, Robertson D, Delia D, et al. Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. *Leukemia* 1992;6 Suppl 1:31-6.
3. Pisacane Pisacane AM, Picciotto F, Risio M. CD31 and CD34 expression as immunohistochemical markers of endothelial transdifferentiation in human cutaneous melanoma. *Cell Oncol* 2007;29(1):59-66.
4. Simmons Simmons DL, Satterthwaite AB, Tenen DG, Seed B. Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. *J Immunol* 1992;148(1):267-71.
5. Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* 2000;151(6):1221-34.
6. Nakamura Y, Komano H, Nakauchi H. Two alternative forms of cDNA encoding CD34. *Exp Hematol* 1993;21(2):236-42.
7. Hemmerich S, Butcher EC, Rosen SD. Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, and adhesion-blocking monoclonal antibody. *J Exp Med* 1994;180(6):2219-26.
8. Kim YC, Wu Q, Chen J, Xuan Z, Jung YC, Zhang MQ, et al. The transcriptome of human CD34+ hematopoietic stem-progenitor cells. *Proc Natl Acad Sci U S A* 2009;106(20):8278-83.
9. Kishimoto S, Ishihara M, Kanatani Y, Nambu M, Takikawa M, Sumi Y, et al. Selective Expansion of CD34+ Cells from Mouse Bone Marrow Cultured on LH/P MP-Coated Plates with Adequate Cytokines. *J Tissue Eng* 2011;2(1):2041731411425419.
10. Nielsen JS, Mcnagny KM. Novel functions of the CD34 family. *J Cell Sci* 2008;121(Pt 22):3683-92.
11. Blanchet MR, Maltby S, Haddon DJ, Merkens H, Zbytnuik L, Mcnagny KM. CD34 facilitates the development of allergic asthma. *Blood* 2007;110(6):2005-12.

12. Hernandez Mir G, Helin J, Skarp KP, Cummings RD, Makitie A, Renkonen R, et al. Glycoforms of human endothelial CD34 that bind L-selectin carry sulfated sialyl Lewis x capped O- and N-glycans. *Blood* 2009;114(3):733-41.
13. Gaipa G, Coustan-Smith E, Todisco E, Maglia O, Biondi A, Campana D. Characterization of CD34+, CD13+, CD33- cells, a rare subset of immature human hematopoietic cells. *Haematologica* 2002;87(4):347-56.
14. Testa JE, Chrastina A, Oh P, Li Y, Witkiewicz H, Czarny M, et al. Immunotargeting and cloning of two CD34 variants exhibiting restricted expression in adult rat endothelia in vivo. *Am J Physiol Lung Cell Mol Physiol* 2009;297(2):L251-62.
15. Wisniewski D, Affer M, Willshire J, Clarkson B. Further phenotypic characterization of the primitive lineage- CD34+CD38-CD90+CD45RA- hematopoietic stem cell/progenitor cell sub-population isolated from cord blood, mobilized peripheral blood and patients with chronic myelogenous leukemia. *Blood Cancer J* 2011;1(9):e36.
16. Colmegna I, Weyand CM. Haematopoietic stem and progenitor cells in rheumatoid arthritis. *Rheumatology (Oxford)* 2011;50(2):252-60.
17. Kelley TW, Huntsman D, Mcnagny KM, Roskelley CD, Hsi ED. Podocalyxin: a marker of blasts in acute leukemia. *Am J Clin Pathol* 2005;124(1):134-42.
18. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem* 2006;54(4):385-95.
19. Mackie AR, Losordo DW. CD34-positive stem cells: in the treatment of heart and vascular disease in human beings. *Tex Heart Inst J* 2011;38(5):474-85.
20. Apiratmateekul N, Phunpae P, Kasinrerk W. A modified hybridoma technique for production of monoclonal antibodies having desired isotypes. *Cytotechnology* 2009.
21. Hollingsworth MA, Rebellato LM, Moore JW, Finn OJ, Metzgar RS. Antigens expressed on NIH 3T3 cells following transformation with DNA from human pancreatic tumor. *Cancer Res* 1986;46(5):2482-7.
22. Kazemi T, Tahmasebi F, Bayat AA, Mohajer N, Khoshnoodi J, Jeddi-Tehrani M, et al. Characterization of novel murine monoclonal antibodies directed against the extracellular domain of human HER2 tyrosine kinase receptor. *Hybridoma (Larchmt)* 2011;30(4):347-53.
23. Klapper LN, Vaisman N, Hurwitz E, Pinkas-Kramarski R, Yarden Y, Sela M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* 1997;14(17):2099-109.
24. Buhring HJ, Sures I, Jallal B, Weiss FU, Busch FW, Ludwig WD, et al. The receptor tyrosine kinase p185HER2 is expressed on a subset of B-lymphoid blasts from patients with acute lymphoblastic leukemia and chronic myelogenous leukemia. *Blood* 1995;86(5):1916-23.
25. Moscatelli D, Quarto N. Transformation of NIH 3T3 cells with basic fibroblast growth factor or the hst/K-fgf oncogene causes downregulation of the fibroblast growth factor receptor: reversal of morphological transformation and restoration of receptor number by suramin. *J Cell Biol* 1989;109(5):2519-27.
26. Nakayasu M, Shima H, Aonuma S, Nakagama H, Nagao M, Sugimura T. Deletion of transfected oncogenes from NIH 3T3 transformants by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci U S A* 1988;85(23):9066-70.
27. Nigro S, Geido E, Infusini E, Orecchia R, Giaretti W. Transfection of human mutated K-ras in mouse NIH-3T3 cells is associated with increased cloning efficiency and DNA aneuploidization. *Int J Cancer* 1996;67(6):871-5.
28. Thorgeirsson UP, Turpeenniemi-Hujanen T, Williams JE, Westin EH, Heilman CA, Talmadge JE, et al. NIH/3T3 cells transfected with human tumor DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. *Mol Cell Biol* 1985;5(1):259-62.
29. Kozak M. The scanning model for translation: an update. *J Cell Biol* 1989;108(2):229-41.
30. Mao M, Fu G, Wu JS, Zhang QH, Zhou J, Kan LX, et al. Identification of genes expressed in human CD34(+) hematopoietic stem/progenitor cells by expressed sequence tags and efficient full-length cDNA cloning. *Proc Natl Acad Sci U S A* 1998;95(14):8175-80.
31. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997;90(12):5013-21.
32. Brown MC, Joaquim TR, Chambers R, Onisk DV, Yin F, Moriango JM, et al. Impact of immunization technology and assay application on antibody performance--a systematic comparative evaluation. *PLoS One* 2011;6(12):e28718.
33. IACUC Policy on Administering Complete Freund's Adjuvant (CFA) and other Adjuvants. [20 June 2014]; Available from: http://www.iacuc.emory.edu/documents/367_Complete_Freunds_Adjuvent.pdf.
34. Furley AJ, Reeves BR, Mizutani S, Altass LJ, Watt SM, Jacob MC, et al. Divergent molecular phenotypes of KG1 and KG1a myeloid cell lines. *Blood* 1986;68(5):1101-7.
35. Hong HY, Sun YX, Guo YX, Wang JN, Lai CN, Qi ZT, et al. Cloning and Expression of Human CD20 Gene on NIH-3T3 Cell Membrane. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 2000;32(4):430-3.