Applied Immunology Series:

A Novel mAb against a Human CD34 Peptide Reacts with the Native Protein on CD34$^+$ Cells

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ABSTRACT

Background: Human CD34 is a transmembrane glycoprotein which is expressed in human hematopoietic stem cells (HSCs) and the small-vessel endothelial cells of a variety of tissues. CD34 plays a critical role as a marker for diagnosis and classification of leukemia. Anti CD34 antibodies are used for isolation and purification of HSCs from bone marrow, peripheral blood and cord blood. **Objective:** To characterize a newly produced monoclonal antibody against a human CD34 peptide. **Methods:** Anti CD34 monoclonal antibody (Clone 2C10-D3) was purified from mouse ascitic fluid and hybridoma cell culture supernatants by affinity chromatography and its immune reactivity was examined by ELISA. The purified antibody was further characterized using Western blot and flow cytometry on TF1 (Human Erythroblast) cell line. **Results:** ELISA experiment revealed that the antibody recognized CD34 peptide. Western blot analysis on TF1 cell lysate confirmed the reactivity of the antibody with a 42 KDa protein. Blocking the antibody with a saturating concentration of specific CD34 peptide resulted in loss of its activity with TF1 lysate in Western blot. The 2C10-D3 antibody reacted with TF1 cells in flow cytometry in a similar manner to a commercial anti CD34 monoclonal antibody. **Conclusions:** Our data suggest that the anti CD34 monoclonal antibody (Clone 2C10-D3) is an appropriate antibody to study the CD34$^+$ cells by flow cytometry and Western blot.

Shams M et al. Iran J Immunol. 2013; 10(4):259-266

Keywords: CD34, ELISA, Flow Cytometry, Monoclonal Antibody, Western blotting

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INTRODUCTION

CD34 is a type I transmembrane glycoprotein (1-2). It is a well-known marker which is expressed on hematopoietic stem/progenitor cells (HSPCs) (3-6). CD34 is also expressed on small-vessel endothelial cells (7-8). It is believed that leukemia cells are derived from immature hematopoietic precursor cells. Therefore, CD34 is an important marker in diagnosis and subclassification of leukemia (6). Anti CD34 antibodies are used for isolation and purification of HSCs for transplantation purposes (9). In order to characterize HSPCs different monoclonal antibodies (mAb) were initially used namely MY10 (3) and B1.3C5 (10-11). These antibodies recognize 2-4% of cells among normal bone marrow leukocytes. These cells were further proved to belong to colony forming cells for myeloid and erythroid lineages and few of them exhibited primitive lymphoid cell properties (12). In addition, later investigations revealed that the aforementioned antibodies could identify a protein (approximately 110-120 KDa) (7,13-14) and the antibodies were proposed to be appropriate for identifying CD34 molecules. However, the different glycosylation patterns in cells (2, 6-7, 12) and alternative splicing of CD34 molecule (12,15-16) results in extensive fluctuation in its molecular weight. In fact, the amino acid sequence deduced from the human CD34 gene sequence predicts a polypeptide of approximately 40 kDa (6-7,12). In this regard, different anti CD34 mAbs have been introduced that react with 42 KDa CD34 molecule (17-18). In 1987, in Seattle, another moAb (clone 12.8) was reported. Not only did this antibody show the expression of CD34 on a variety of lineage-committed progenitors, but it also detected the expression of CD34 on hematopoietic stem cells which have a role in long-term multi-lineage hematopoiesis (19).

Preliminary studies on human bone marrow transplantation manifested that the CD34 positive stem cells are more efficient than the negative ones in transplantation (20). Several clinical examinations also proved that $CD34^+$ transplants are more reliable (21). With increasing the number of $CD34^+$ stem cell transplantation cases, there is an increasing need for newer reagents and facilities. In this regard, here we report characterization of a novel murine anti CD34 mAb that was originally raised against a CD34 specific peptide.

MATERIALS AND METHODS

Production and Affinity Purification of Anti-CD34 mAb (Clone 2C10-D3). Using hybridoma technology (22) a monoclonal antibody, clone 2C10-D3 was primarily produced against a human CD34 peptide (CAEFKKDRGEGLARVL). The antibody was then produced in Balb/c mouse ascitic fluid by injecting 5×10^6 2C10-D3 hybridoma cells into the peritoneal cavity of the previously pristaned mice (Sigma ALDRICH- Missouri-USA). The ascitic fluid was then collected and applied onto Benzamidine column (Hitrap, GE Healthcare, Uppsala, Sweden) to remove serine proteases, and then the antibodies were purified using a HiTrap protein G HP affinity chromatography column (GE Healthcare) using PBS (0.15 M, pH 7.2) and Glycine-HCl (0.1 M, pH 2.5) as binding buffer and elution buffer, respectively. The purified antibody was dialyzed against PBS overnight at 4°C. Finally, the concentration of the purified antibody was estimated by measuring its absorbance at 280 nm using a biophotometer

(Eppendorf, Hamburg, Germany). The isotype of the antibody was determined to be IgG2a using Isostrip determination kit (Roche, Basel, Switzerland).

Purity Assessment of the Purified 2C10-D3 mAb. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity and molecular weight of the anti CD34 antibody under non-reducing conditions. In this regard, the 2C10-D3 mAb and protein marker including Human IgG and bovine serum albumin (BSA) were loaded onto 10% resolving gel and electrophoresed using a Mini Protean Tetra electrophoresis apparatus (Bio Rad, Berkeley, California, USA). The gels were then stained with Coomassie Brilliant Blue dye for 60 min followed by destaining until protein bands appeared against a destained gel background.

Immune Reactivity of Purified 2C10-D3 mAb with the Immunizing Peptide. Ten µg/ml of CD34 peptide in 100 µl volume were coated on each well of microtiter polystyrene plate (Maxisorp, Nunc, Roskilde, Denmark) and incubated at 4°C overnight. The plate was then washed with PBS-Tween-20 (0.05%) (PBS-T) three times. Then, it was blocked with 2.5% skim milk at 37°C for 1.5 hr. After three times washing with PBS-T, serial dilutions of the antibody starting from 10 to 0.625 µg/ml were added to the wells (100 µl) and incubated for 1.5 hr at 37°C. After washing, Sheep anti mouse-HRP (Avicenna Research Institute, Tehran, Iran) was added as secondary antibody for 1 hr at 37°C. After five times washing, tetramethylbenzidine (US Biological, Marblehead, MA, USA) was added to the wells; and then, the plate was incubated at RT in the dark. After 2 min incubation, the reaction was stopped by adding 30 µl of H2SO4 (20% V/V) into each well. This procedure was also performed for an irrelevant peptide as negative control. Finally, the absorbance was measured by an ELISA reader (BioTek, Winooski, VT, USA).

Western Blot Analysis and Peptide Inhibition Assay. TF1 ($CD34^+$ Human Erythroblast) (23-24) and Raji ($CD34^-$ B-Cell Burrkitt's lymphoma) (23,25) cell lines were lysed using ice-cold lysis buffer (10 mM Tris-HCL pH 8, 130 mM NaCl, 5 mM EDTA, and Triton X-100 (1% v/v) with Protease Inhibitor (PI) (Roche, Basel, Switzerland) and Phosphatase Inhibitor Cocktail (PIC) (Sigma ALDRICH- Missouri-USA). Twenty µg/well of each cell lysate were loaded on a 10% SDS-PAGE gel. After electrophoresis under non-reducing conditions and transferring proteins onto PVDF membrane (Millipore, Billerica, MA, USA), the membrane was blocked with 5% non-fat skim milk in PBS containing 0.1% Tween-20 at 4^oC overnight. After gentle washing with PBS-T, the 2C10-D3 mAb (10 µg/ml in PBS-T and 3% skim milk) was added to the membrane and incubated for 1.5 hr at RT with shaking. After 6 times (10 min incubation interval) washing, the membrane was incubated with Sheep anti mouse-HRP (1:2500 dilution in PBS) for 1.5 hr at RT with shaking. After washing as above, the membrane was exposed to Luminata Forte Western HRP substrate (Merck Millipore, Billerica, Massachusetts, USA) followed by autoradiography on X-Ray film (KODAK Medical X-Ray film, Z&Z Medical, Iowa, USA).

To validate the protein band specificity detected in Western blot, the 2C10-D3 mAb reactivity was blocked with a saturating concentration of the immunizing peptide (40:1 peptide to antibody molar ratio) (26). In this regard, the peptide was added to the 2C10-D3 mAb for 1hr at 37^oC and then the mixture was added to the PVDF membrane, as explained above. Unblocked 2C10-D3 mAb was added in parallel to another PVDF (same as the former PVDF) to be used as positive control. The incubations, washings and development of bands were performed as above.

Flow Cytometry Study of 2C10-D3 mAb. Flow cytometry analysis was performed to survey the cell surface reactivity of 2C10-D3 mAb on $CD34^+$ TF1 and $CD34^-$ Raji cells. In this regard, TF1 and Raji cells (10^6) were suspended in cold PBS and blocked with 5% sheep serum for 15 min. Cells were then incubated with 2C10-D3 mAb (10 µg/mL), an irrelevant isotype control, Anti ENV (10 µg/mL, Avicenna Research Institute, Tehran, Iran) and a commercial positive control (QBEND-10, Abcam, 1:50) for 1.5 hr at 4°C. All antibodies were diluted in PBS containing 1% BSA. After 3 times washing with cold PBS, Sheep anti mouse-FITC (Avicenna Research Institute, 1:50) were added to the cells for 45 min in the dark at 4°C, followed by washing. Cells were then analyzed using a flow cytometer (Partec, Munster, Germany).

To analyze the potential of the immunizing peptide to interfere with antibody binding to CD34 on cell surface and to compare the epitope recognized by 2C10-D3 with that of a commercial anti-CD34 antibody, 2C10-D3 mAb was conjugated with FITC (27) and then blocked with a saturating concentration of the immunizing peptide (40:1 peptide to antibody molar ratio) (26). Likewise, a FITC-conjugated anti-CD34 commercial antibody clone 581/CD34 (BD Bioscience, New Jersey, USA) was used in a similar blocking assay. Flow cytometry was then performed using both peptide-blocked and unblocked antibodies on TF-1 cells as explained above.

RESULTS

Confirming the Purification of 2C10-D3 mAb by SDS-PAGE. Figure 1 shows a single band of around 150 KDa on the SDS-PAGE gel, representing the purity of the purified 2C10-D3 mAb. Lack of degraded protein bands suggests that the purification procedure did not result in any breakage in the antibody structure.

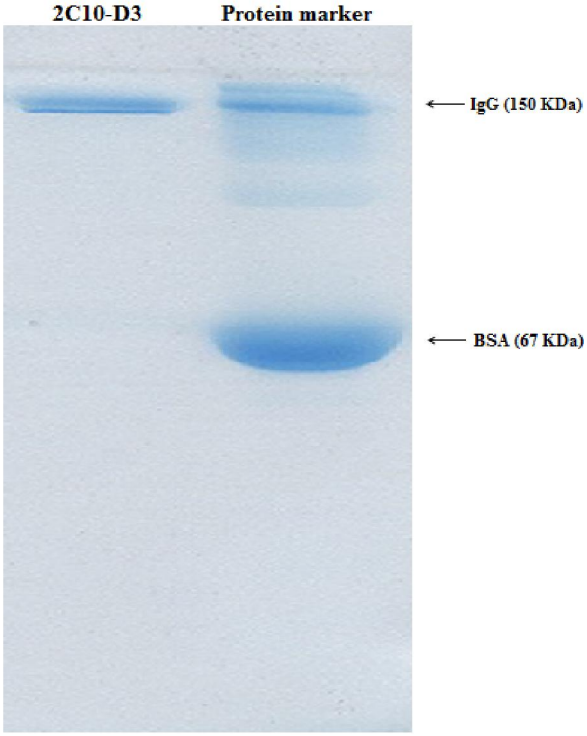

Figure 1. SDS-PAGE analysis of 2C10-D3 mAb. BSA and human IgG were used as protein markers.

Immune Reactivity of 2C10-D3 mAb with Specific Peptide. ELISA assay presented in Figure 2 showed that the purified 2C10-D3 mAb maintained its activity with CD34 peptide after purification procedure. In addition, the antibody showed no reactivity with irrelevant peptide, confirming its specifity for CD34 peptide.

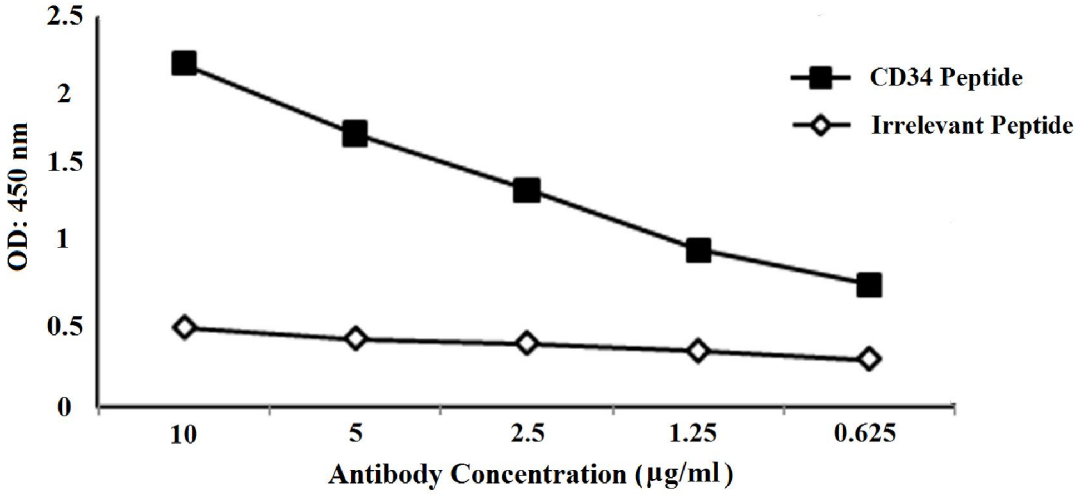

Figure 2. Immune reactivity of 2C10-D3 mAb with CD34 specific peptide ar ( ) relevant peptide in E( )

Western Blot Analysis and Peptide Inhibition Assay. Western blot analysis showed that the 2C10-D3 mAb could recognize CD34 molecules around 42 KDa in TF1 cell lysate while it showed no reactivity with Raji cell lysate (Figure 3).

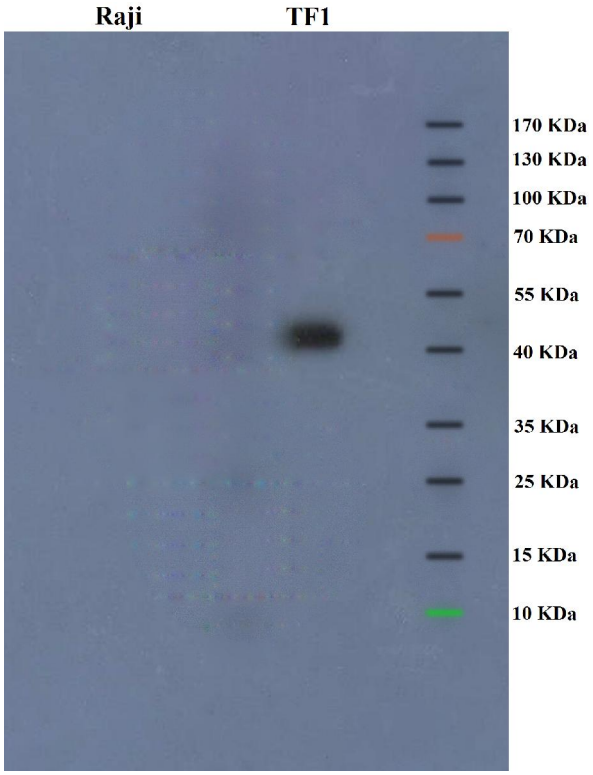

Figure 3. Western blot analysis of 2C10-D3 mAb reactivity with TF1 ($CD34^+$) and Raji ($CD34^-$) cell lysates.

Additionally, blocking the 2C10-D3 mAb with CD34 peptide resulted in loss of its activity with TF1 lysate, confirming its reactivity with the CD34 molecule (Figure 4).

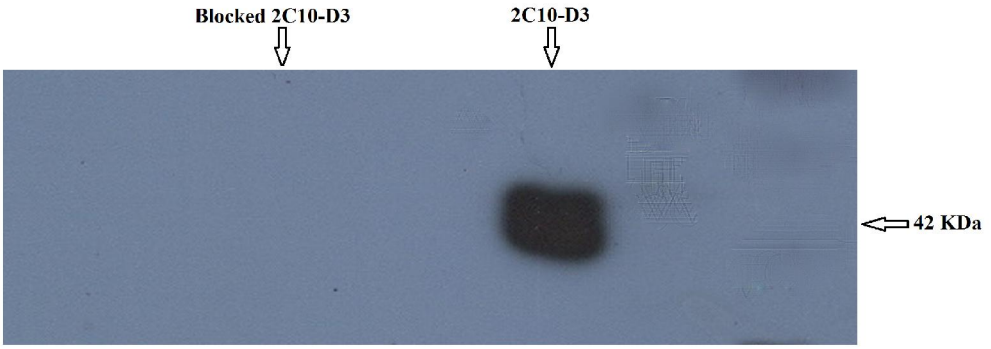

Figure 4. Western blot analysis of CD34 peptide-blocked 2C10-D3 and unblocked 2C10-D3 mAb on TF1 cell lysates.

Flow Cytometric Analysis and Peptide Inhibition Assay. In flow cytometric analysis, 2C10-D3 mAb stained the surface of TF1 cells in a similar manner to the commercial anti CD34 antibody (94.4% vs. 98.7%) while both antibodies were negative on surface of Raji cells (2.6% vs. 1.1% for 2C10-D3 mAb and commercial antibody, respectively) (Figure 5). Furthermore, blocking the 2C10-D3 and the commercial antibody with CD34 peptide resulted in loss of binding of both antibodies to TF1 cells, confirming 2C10-D3 specificity for the CD34 molecule.

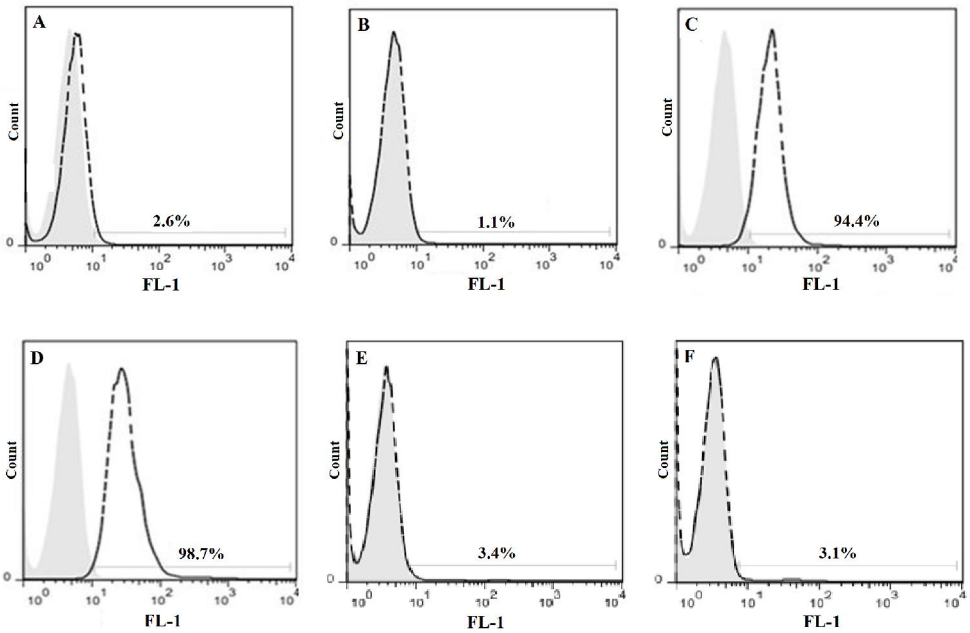

Figure 5. Flow cytometric analysis of 2C10-D3 mAb reactivity with Raji (A) and TF1 (C) cell lines. Reactivities of a commercial anti CD34 mAb with Raji (B) and TF1 (D) cell lines are demonstrated for comparison. Blocking of 2C10-D3 and the commercial antibody with CD34 specific peptide resulted in loss of binding of both antibodies to TF-1 cells in flow cytometry, panels (E) and (F), respectively. Filled zones demonstrate isotype control antibodies and dashed lines represent anti CD34 antibodies.

DISCUSSION

The CD34 molecule has gained special attention in research and clinic after it was found to be expressed on a fraction of human bone marrow cells (3-6). Anti CD34 antibody is used in isolation and purification of HSPCs for transplantation (9) and a wide variety of research applications, including sub-classification and diagnosis of hematological malignancies, estimation of prognosis and follow up of therapy against leukemia and studies about homing, survival, migration and differentiation of hematopoietic and endothelial progenitor cells. Administration of cytoreductive drugs, such as cyclophosphamide (28-29), and cytokines, such as granulocyte colony-stimulating factor (G-CSF) (29-30) has been shown to mobilize $CD34^+$ cells from the bone marrow to periphery. This accumulation of $CD34^+$ cells in blood, as studied by flow cytometry, will enable the physicians to estimate the optimum time for blood sample collection for the best $CD34^+$ cell recovery for transplantation purposes (29). $CD34^+$ cells can be purified from bone marrow, cord blood and Peripheral Blood (PB) (31). In order to find an appropriate anti CD34 monoclonal antibody, we characterized the newly produced Clone 2C10-D3. The 2C10-D3 mAb class was determined to be IgG2a, thus, Protein G affinity column was used to purify the antibody. The antibody reactivity with CD34 was confirmed by Flow cytometry, ELISA, Western blot and blocking with specific peptide.
There are two mRNA splice variants of murine and human CD34 genes that are responsible for encoding a full-length (Isoform-F) and a truncated (Isoform-T) form of the protein (15). The number of amino acids in full-length and truncated forms of human CD34 molecule are 385 and 328 (32), and thus the predicted band sizes are around 42 and 36 KDa, respectively (33). Based on different glycosylation patterns (2, 6-7,12) and alternative splicing (12,15-16) various forms of the CD34 molecule with different molecular weights may be produced, although it is not clear whether differential glycosylation can account for these differences (33).
Enrichment of $CD34^+$ cells is gaining more importance due to its application in transplantation. In this regard, conjugation of 2C10-D3 antibody to HRP enzyme, FITC and immunomagnetic particles, will materialize its direct use in Western blot, flow cytometry and immunomagnetic methods, respectively.
In summary, our results suggest the 2C10-D3 mAb as a proper antibody for use in flow cytometry and Western blot experiments as well as a tool for immunomagnetic separation of $CD34^+$ cells.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Avicenna Research Institute and Tehran University of Medical Sciences. We are grateful to the technical staff of the Antigen-Antibody Engineering and Immunochemistry Department of Avicenna Research Institute for their invaluable cooperation.

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