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制备和表征抗CD34单克隆抗体，这些抗体可以识别造血干细胞

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Abstract

CD34是一种膜蛋白，其分子量约为110 kDa。该抗原与人造血祖细胞相关，是一种分化阶段特异性的白细胞抗原。在本研究中，我们已制备和表征了针对CD34标记的单克隆抗体（mAbs）。用二价血蓝蛋白（KLH）结合CD34多肽对小鼠进行免疫接种。融合细胞在选择性培养基（HAT）中生长，并通过稀释极限（L.D）法进行克隆。从这些中，我们选择了稳定的克隆，这些克隆可持续产生抗体。这种抗体被测试以识别CD34多肽，并且进一步通过酶联免疫吸附法（ELISA）和Western blot方法进行筛选。其中一个抗体（3D5）在ELISA和Western blot分析中对CD34多肽具有高度的特异性和功能性。这些单克隆抗体（mAbs）可以作为生物医学应用的有用工具，用于人造血干细胞（HSCs）的分离和纯化。

Keywords: Monoclonal Antibody, CD34, Hematopoietic Stem Cells, Isolation

CD34是一种单链跨膜糖蛋白，分子量约110 kDa。该抗原与人类造血祖细胞相关，是分化阶段特异性的白细胞抗原。在本研究中，我们已制备和表征了针对CD34标记的单克隆抗体（mAbs）。用二价血蓝蛋白（KLH）结合CD34多肽对小鼠进行免疫接种。融合细胞在选择性培养基（HAT）中生长，并通过稀释极限（L.D）法进行克隆。从这些中，我们选择了稳定的克隆，这些克隆可持续产生抗体。这种抗体被测试以识别CD34多肽，并且进一步通过酶联免疫吸附法（ELISA）和Western blot方法进行筛选。其中一个抗体（3D5）在ELISA和Western blot分析中对CD34多肽具有高度的特异性和功能性。这些单克隆抗体（mAbs）可以作为生物医学应用的有用工具，用于人造血干细胞（HSCs）的分离和纯化。

CD34表达在各种肿瘤中，包括血管肿瘤和急性淋巴细胞白血病（5）。该分子最初被识别为CD34阳性的造血干细胞（HSCs），现已广泛用于分离和纯化生物医学应用的HSCs。

目的：本研究的目的是制备并表征针对CD34单克隆抗体（mAbs），这些抗体可以用于分离和纯化造血干细胞。
Generation of anti-CD34 mAbs reactive with HSCs

For this study, the amino acid sequence of human CD34 was carefully analyzed. Then, two 14-mer synthetic peptides that sequenced TF-SNVSTNVSYQET and NTNSSVQSQTSVIS from the extracellular portion of the human CD34 protein were designed and selected as the immunogen, based on local hydrophilicity as predicted by the method of Hopp and Woods (7). The designed peptide was separately conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) (Thermo, USA), following the procedure provided by the manufacturer (8). At the next step we used four, 6-week-old Balb/c female mice for peptide immunization. All animal experiments in this research followed the guidelines of the Laboratory Animal Ethical Commission of Tabriz University of Medical Sciences. Each mouse was immunized 4 times over a 2-3 week interval. One week after the last immunization, blood was taken from each mouse by a vertical incision of the tail vein (after anesthesia with ether for pain prevention) and the antibody response was measured by enzyme-linked immunosorbent assay (ELISA) as described previously (9). The mouse with the highest serum antibody titer was selected as the spleen donor. Next, to collect mouse peritoneal macrophages as a feeder layer, we injected RPMI-1640 media into the peritoneal cavity of an unimmunized Balb/c mouse followed by subsequent aspiration and collection of the peritoneal cells. Mouse myeloma SP2/0 cell line was used as the fusion partner. Thus, one week before fusion cells were cultured in RPMI (Gibco) and 10% FBS until they attained >70% confluency in the logarithmic phase. The spleen cells of the immune mouse were removed under sterile conditions. Spleen cells were fused with SP2/0 cells at a 5 to 1 ratio by PEG1450 (Sigma-Aldrich Co. St. Louis, MO, USA) as the fusogen. Supernatants of the growing wells were screened for the production of antibody using an ELISA method as described previously (9).

After screening, clones that had high absorbance were selected for cloning by the limiting dilution (L.D) method. Suitable monoclonals that possessed high absorbance were selected for characterization of antibodies. The class and subclass of mAbs were determined by an ELISA with a mouse monoclonal subclassotyping kit that contained rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA following the procedure provided by the manufacturer (Thermo, USA) (9). At the next step, the mAbs were purified from culture supernatants using sepharose beads conjugated with protein A column affinity chromatography according to isotype. Confirmation of the mAb purity was monitored by SDS-PAGE under a non-reducing condition.

We next attempted to determine whether these antibodies were capable of identification and ultimate enrichment for hematopoietic stem/progenitor cells. To accomplish this, we used Western blotting according to the protocol we described elsewhere with minor modification (10). Initially, umbilical cord samples were obtained from the umbilical vein after the vaginal delivery of normal-term babies following informed consent by the healthy mothers. Then, mononuclear cells (MNC) were isolated by ficoll-hypaque (1.077 g/ml, Pharmacia Biotech) density gradient centrifugation (10). Next, we choose a panel of different cell lines with origin of blood such as Raji and HL-60 for the cross-reactivity assay. In addition, cells were cultured in their recommended medium, harvested and lysed with lysis buffer. The protein concentration of lysate was measured by a biophotometre (Ependorff, Germany). The samples were loaded onto a 12.5% SDS-PAGE gel at 100 V for 2 hours. After electrophoresis, the SDS-PAGE gel was transferred electrophoretically to wet nitrocellulose membrane. Transfer of proteins from the gel to a nitrocellulose membrane was undertaken at 100 mA for 2 hours. Then, the membrane was developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech Inc., USA).
In this study, we used the peptide-KLH for mice immunization and peptide-BSA for conjugation assessment and specificity testing of the antibody. Due to the very high molecular weight of KLH, it is not possible to run the KLH conjugate on SDS-PAGE. In this context, BSA conjugate was used for efficacy of conjugation. The coupling efficiency, as determined by the SDS-PAGE peptide was suitable. Four mice were immunized four times against KLH-conjugated peptides. Then, we evaluated sera from the mice in direct binding assays for antibody reactivity with BSA-peptide conjugate. Serum of the immune mouse at the 1:8000 dilution displayed a high absorbance in reaction with BSA-peptide by indirect ELISA (Fig 1). Accordingly, the mouse with a higher titer of specific antibody (Mouse 3) was selected for hybridoma production. Spleen cells from the immune mouse were fused with myeloma SP2/0 as the fusion partner (Fig 2A). The fused cells were suspended in hypoxanthine, aminopterine and thymidine (HAT) medium and distributed into five culture plates that contained feeder layer (Fig 2B).

Several anti-CD34 mAbs that produced hybridomas were obtained. From these, the 3D5 clone showed high reactivity with immunogenic peptide in the ELISA assay (Figs 2C, D). For this reason, we performed all subsequent tests with this clone. Further characterization of this antibody showed that it was an IgG1 isotype with a kappa light chain (Table 1). We assessed purity of this antibody by SDS-PAGE. A single band of approximately 150 kDa in SDS-PAGE analysis indicated the proper purification of the antibody (Fig 3A).

Fig 1: Evaluation of anti-CD34 antibody production with immunizing peptide in the serum of immunized mice by ELISA. The serum of the immune mouse and the non-immune mouse negative control were diluted 1:8000. NC; Negative control, M1; Mouse 1, M2; Mouse 2, M3; Mouse 3, M4; Mouse 4 and OD; Optical density.
Generation of anti-CD34 mAbs reactive with HSCs

Fig 2: Proliferated monoclonal with higher optical density as the suitable monoclonal. A. Sp2/0 in logarithmic phase., B. Mouse peritoneal macrophages as feeder layer, C. Monoclonal in the highly proliferated form (magnification: ×20), and D. Monoclonal in the growing form (magnification: ×40).

Table 1: ELISA mouse monoclonal antibody (mAb) isotyping
Class and subclass monoclonal antibodies of the 3-D5 monoclonal

| Class     | IgG1 | IgG2a | IgG2b | IgG3 | IgA  | IgM  | Kappa | Lambda |
|-----------|------|-------|-------|------|------|------|-------|--------|
| Clone 3-D5| 1.021| 0.156 | 0.154 | 0.109| 0.157| 0.131| 1.632 | 0.144  |

*ELISA*: Enzyme-linked immunosorbent assay.
In addition, the purified antibody showed immunoreactivity with the immunizing peptide in ELISA. Western blotting technique was performed to see the pattern of reactivity of anti-CD34 mAbs with different cell lines such as Raji, HL-60, and umbilical cord blood (UCB)-derived CD34 cells. Only one specific band was visualized in 110 kDa in the UCB lysate (Fig 3B). There was good concordance between the results obtained in both the ELISA and Western blot assays. Taken together, these results illustrated that this antibody was highly specific and functional in applications such as ELISA and Western blot assays.

Here, we have employed a peptide-based antibody generation protocol for producing antibody against human CD34 using a new immunization strategy. The use of synthetic peptides as immunogens is generally applied when either the complete protein is not available in sufficient quantities to carry out an adequate immunization protocol or to obtain antibodies that have the capability to recognize only specific regions of a polypeptide chain (11). Synthetic peptides offer the opportunity for a very fast shortcut to overcome a lack of protein. The secondary and tertiary configuration of the peptides, their length, hydrophilicity and location in the native molecule may all be important factors in generating useful antibodies (12). One problem with utilizing a peptide-based antigen is that, because of their small size, peptides are not likely to elicit a robust stimulation of the immune system. KLH has been shown to be an effective carrier protein for immunization with short peptides in the high-yield production of antibodies for research, biotechnology and therapeutic applications (13).

Until now, a large series of CD34-specific mAbs have been developed. Civin et al. produced and developed high affinity murine monoclonal antibodies (My10) that recognized CD34 with a high affinity for diagnosis of hematopoietic progenitor cell. KG-1a cells that possess a large amount of CD34 have been used for immunization (14). The QBEnd antibody, which belongs to class II CD34 epitope mAbs, has been used to isolate HSCs (15).

In conclusion, anti-CD34 mAb can be used in the diagnosis of hematologic malignancies, solid tumors, and immunodeficiency diseases, isolation of hematopoietic progenitor cells, disease monitoring, and in vitro differentiation studies. Anti-CD34 mAb may represent a powerful tool for the positive selection or depletion of cells that express human CD34 antigen. Upon our findings, it can be proposed that this particular approach for production of an anti-CD34 peptide antibody is feasible and cost-effective. This study clearly indicates that the produced antibodies can be used in research and diagnosis as well as clinical applications if produced in the chimeric form.

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