Isolation And Culture of Rat Intestinal Mucosal Microvascular Endothelial Cells Using Immunomagnetic Beads And Their Marker Expression

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Abstract

Microvascular endothelial cells (MVECs) have been an important tool in many research fields, while their purification remained challenging. This study aimed to establish a method for isolating rat intestinal mucosal MVECs using immunomagnetic beads, and characterize their proliferation ability and marker expression. Rat jejunum mucosae were scraped and digested by collagenase type II to obtain primary cell culture by the differential adhesion method. Magnetic beads were coated with anti-CD31 antibodies and incubated with the single-cell suspension of the primary cell culture. The CD31⁺ cells were separated using an automatic magnetic separation system, and their proliferation ability was assayed at different passages. The expression of factor VIII (FVIII), CD31, and CD34 was detected by immunofluorescence staining. Results indicated that rat intestinal mucosal MVECs grew into a contact-inhibited cobblestone-like monolayer after about 6 days, whose proliferation ability had no significant decline at passage 20. Three markers were generally expressed in the cells, and the fluorescence intensity of FVIII was higher after magnetic separation than before, and those of CD31 and CD34 remained similar. In conclusion, highly pure MVECs can be isolated from the primary cell culture of rat intestinal mucosae using magnetic beads coated with anti-CD31 antibodies, and magnetic separation may influence the expression of FVIII.

Introduction

Endothelial cells in vitro have been the important tool to investigate vascular functions in many fields^{1,2}, while cells from different tissues or organs need to be isolated and cultured due to their high heterogeneity³. The intestinal mucosa is the body's vital defensive barrier, where microvascular endothelial cells (MVECs) are involved in various physiological processes or pathologic responses⁴. Since rat intestinal mucosa is a fragile thin tissue composed of various kinds of cells, it is difficult to isolate pure MVECs from it. Previous reports on rat intestinal mucosal MVECs were mainly in our team's publications⁵⁻⁸, and their isolation and culture were performed as the methods dealing with other tissues or organs^{9,10}. However, the procedures were tedious and had high technical requirements for the experimenters, and contaminating cells grew so rapidly that long-term subculture was impossible.

Immunomagnetic-bead cell separating is a highly specific and sensitive technique, and has been used to isolate MVECs from many tissues and organs, such as rat brains¹¹, mouse lungs¹², and human intestinal mucosae¹³. Although a variety of antibodies or ligands, such as anti-CD44¹⁴, CD144¹², and CD34 antibodies¹⁵, or Evonymus europaeus agglutinin (EEA)¹⁶ and Ulex europaeus agglutinin I (UEA I)¹⁷, had been used to coat the beads, anti-platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31) were the most common one¹⁸. The target cells were separated from the single suspension of the tissues or the cell culture in vitro, which was generally operated using a simple manual separator rather than the automatic magnetic separation system. What effects the procedure of magnetic separating had on MVECs remained unclear, though it was documented that magnetic fields could remarkably influence endothelial functions^{19,20}. The purpose of this study was to establish a culture system of pure MVECs from rat

intestinal mucosas using magnetic beads coated with anti-CD31 antibodies and understand the effects of magnetic separation on the expression of three endothelial markers.

Materials And Methods

Reagents. Fetal bovine serum (FBS, Cat. #10099-141), DMEM (Cat. #11995-065), and Cellaction pan mouse IgG kit (Cat. #11531D) were purchased from ThermoFisher (Waltham, MA, USA). Rabbit anti-Factor VIII antibody (Cat. #11778-1-AP) and FITC-conjugated goat anti-rabbit IgG (Cat. #SA00003-2) were obtained from Proteintech (Wuhan, China). Mouse anti-CD31 antibody (Cat. #MCA1334G) was from Bio-Rad (Hercules, CA, USA). Rabbit anti-CD34 antibody (Cat. #ab185732) was from Abcam (Cambridge, MA, US). Collagenase type II (Cat. #4176) was from Worthington (Lakewood, NJ, USA). Hank's solution (Cat. #H1045), L-Glutamine solution (Cat. #G0200), Trypsin-EDTA solution (Cat. #T1300), 4',6-diamidino-2-phenylindole (DAPI, Cat. #C0065), and 4% Paraformaldehyde (Cat. #P1110) and MTT cell proliferation and cytotoxicity assay kit (Cat. #M1020) were from Solarbio (Beijing, China).

Cell isolation and culture. About 10-day-old Sprague-Dawley rats were purchased from Beijing Haidian Xinglong Experimental Animal Breeding Factory (animal license No.: SCXK [Jing] 2019-0010) and used for cell culture. The experimental protocol for cell isolation from rat intestinal mucosas was approved by the Animal Care and Protection Committee of Beijing University of Agriculture (No. BUA_ZT202001). All procedures were performed in accordance with the relevant guidelines and regulations by Beijing Association on Laboratory Animal Care and the ARRIVE guidelines. Isolation and culture of rat intestinal mucosal MVECs were performed as previously described with minor modification⁵.

In brief, rats were euthanized by cervical dislocation, and the abdominal cavities were opened. The jejuna were sampled and then cut into small segments, which were longitudinally cut and rinsed away the contents with Hank's solution. The intestinal mucosas were scraped and minced in 0.2% collagenase type II solution. After about 60-min digestion at 37°C with pipetting every 15-20 min, the digested solution was filtered through a 100-µm cell strainer and centrifuged. The cell pellet was resuspended in DMEM containing 15% FBS, 2 mmol/L glutamine, 100U/mL penicillin, and 100 mg/mL streptomycin, and the cells were seeded at a density of 5×10^4 cells/mL and incubated in a 5% CO₂ 37°C incubator. After 2 hr of incubation, the nonadherent cells were washed away and the complete medium was replaced. When it grew to confluence, the cell culture was detached using 0.05% trypsin-0.005% EDTA and purified by magnetic separation as follows.

Magnetic cell separation. Magnetic cell separation was carried out using a Cellaction pan mouse IgG kit via the direct technique with the KingFisher 96 automatic magnetic separation system. Briefly, the beads were washed and incubated with mouse anti-CD31 antibodies on a rotary shaker at 4°C overnight. Removing excess antibodies, the beads were resuspended in Buffer 1 (0.01 M PBS containing 0.1% BSA, pH 7.4) of the same original volume. The detached cells were filtered through a 40-µm cell strainer to prepare the single-cell suspension at a density of 1×10^7 cells/mL in Buffer 2 (0.01 M PBS containing 0.1% BSA and 2 mM EDTA, pH 7.4). Twenty-five microliters of CD31 antibody-coated beads were added

into 1 mL of the cell sample. Over 30-min incubation on the rotary shaker at room temperature, CD31⁺ cells were separated using the magnetic separation system. The bead-bound cells were placed into Buffer 3 (DMEM with 1% FBS, 1mM CaCl₂, and 5 mM MgCl₂, Ph 7.2), and the DNase I solution was added according to the manufacture's recommendation. The beads were released over 15-min incubation and removed using the magnetic separation system. The released cells were resuspended in the complete medium and seeded with the medium replacement every 3 days.

Parameter setting of the separation system. The KingFisher 96 automatic magnetic separation system (Kingfisher 96, ThermoFisher Scientific, Vantaa, Finland) was used to wash beads, separate cells, and remove the beads, respectively, which included the KingFisher 96 Magnetic Particle Processor and the BindIt 3.2 operating software. All procedures were processed in KingFisher 96 Deep-Well plates (Cat. #97002534), and five plates were used for beads washing and cells separating and two plates for beads removing. When washing, Beads were released quickly and mixed fast for 5 sec. When separating cells, beads were released and mixed slowly for 5 sec. When removing beads, the sample was shaken strongly for 5 sec and then mixed fast for 5 sec. In every procedure, the beads were collected 3 times for 3 sec each time.

Cell proliferation assay. The cell proliferation ability was assayed at passage 5, 10, and 20. To determine the proliferation parameters, cells were seeded at a density of 5,000 cells per well in a 96-well plate, and the medium was replaced every 3 days. The cell proliferation was determined each day in quintuplicate by the MTT method according to the kit instruction. To determine the number parameters, cells were plated at a density of 50,000 cells in a corning 12 well plate, and the medium of 1 mL per well was replaced every 3 days. Cells were dissociated in triplicate by trypsin digestion each day and counted using a cell counter (TC20, Bio-Rad). The cell growth curves were drawn from the absorbance values or cell numbers for 7 days.

Immunofluorescent staining. Cells were grown on cover glasses in 24-well plates for 48 h and fixed in 4% paraformaldehyde for 20 min at room temperature. The expression of CD31, CD34, and FVIII were detected using indirect immunofluorescent staining as described previously¹⁴. Briefly, after washing with PBS three times, the fixed pieces were blocked with 1% bovine serum albumin for 30 min and incubated with primary antibodies for CD31, CD34, or FVIII in blocking solution (10 µg/mL) overnight at 4°C. The cell pieces were washed with PBS three times and incubated with FITC-labeled secondary antibody in blocking solution (1:100 dilution) for 2 h at room temperature in the dark. After washing, cell nuclei were counterstained with 2 µg/mL DAPI for 3 min. The stained cells were mounted in glycerol and photographed using a fluorescence microscope (IX71, Olympus, Tokyo, Japan) with 490 and 350-nm excitation, and then the single-channel images were merged. Their fluorescence intensities were measured using Image J software.

Data analysis. Data were presented as the mean ± standard deviation with three or more replicates. Statistical comparisons were performed by one-way ANOVA combined with Tukey's post hoc multiple comparison test using GraphPad Prism 8.0 software, and $P < 0.05$ was considered a significant difference.

Results

Isolation of MVECs by immunomagnetic beads. To obtain pure MVECs from rat intestinal mucosas, the microvascular endothelial cell fraction was firstly enriched by the traditional collagenase digestion and differential adhesion method. One plate of primary cell culture was grown from the jejunum mucosas of two rats, which was dissociated into the single-cell suspension (Fig. 1A) and incubated with about 2×10^7 beads coated with anti-CD31 antibodies (Fig. 1B). A target cell could bind a few to dozens of beads (Fig. 1C, 1D), and some cells cross-linked via immunomagnetic beads to form cell masses. About ten percent of CD31⁺ cells were separated from the primary cell culture, and the beads-released cells grew with adherence in good condition.

Morphology and growth characteristics. The separated MVECs grew fast into a monolayer. Compared with the primary cell culture, the confluent separated MVECs displayed a more uniform cobblestone-like morphology (Fig. 2). The cell viability assay by the MTT method and proliferation characteristic by the number counting method indicated similar results (Fig. 3). After 2 days post-seeding, the numbers of MVECs began to increase for about 3 days in a logarithmic fashion, and then cells entered a state of contact inhibition. Their proliferation abilities had no significant decline from passage 5-20, and cells still possessed the logarithmic growth performance at passage 20.

Expression of endothelial markers. To identify MVECs and understand effects of immunomagnetic separation on their marker expression, three endothelial markers were detected in primary cell culture and separated MVECs by immunofluorescence staining. As shown in Fig. 4, the positive staining of FVIII, CD31, and CD34 was determined in both cell cultures, while the percentage of positive cells and their mean fluorescence intensities were different. Statistical analysis indicated that all the positive cell percentages of three markers extremely significantly increased to over 95% ($P < 0.01$), and the fluorescence intensity of FVIII in separated MVECs was significantly higher than that in the primary cell culture (Fig. 5). The fluorescence intensities of CD31 and CD34 had no significant changes before and after magnetic separation.

Discussion

This study aimed to separate pure MVECs from rat intestinal mucosas using immunomagnetic beads coated with anti-CD31 antibodies and to understand the effects of magnetic separation on the expression of endothelial markers. The results show that MVECs were obtained by magnetic separation from primary cell culture of rat jejunal mucosas and their purity was over 95%, which could be subcultured at least 20 passages, and that out of three markers, expression of FVIII was remarkably increased after immunomagnetic separation.

The intestinal mucosas contain not only large amounts of non-adherent cells, such as blood cells and mucosal lymphocytes, but also lots of acellular components, which would disturb the binding of beads to target cells. Thus, the single-cell suspension sample was prepared from the primary cell culture rather

than the digested mucosae, and the tissue blocks were well avoided. Moreover, the proportion and quantity of target cells were enhanced in the primary cell culture due to the differential-adhesion handling and the cell proliferation.

The expression of CD31 in rat intestinal mucosae had been well documented²¹, so in this study the anti-CD31 antibodies were used to coat the beads. However, not all commercial mouse anti-rat CD31 antibodies are available for immunomagnetic separation, which is generally not marked in their applications. Since the antibodies would bind the living cells, we considered that they should be derived from natural or recombinant proteins rather than from synthetic peptides. The antibodies for flow cytometry should be better than those for western blot or immunocytochemistry, which had been proved in our research. Here a high-affinity antibody was selected from four alternates, and it worked well via the direct immunomagnetic technique.

Unlike most studies using simple magnets^{22,23}, the automatic magnetic separation system was used in our study, which is high throughput equipment and has a better separation effect. Owing to that the magnetic rods with the combs were into the sample suspension, the unbound-beads cells couldn't be retained as the cell suspension flowed through the separation column, and on the other hand, the positive cell masses could be separated. When setting the protocols of the separation system, the mixing speed is the parameter that should be paid attention to. Slow or medium mixing is preferable to fast mixing for washing beads-bound cells on account of avoiding dissociating them and breaking cells, and fast mixing can promote the separation of beads and cells and is preferable for releasing beads.

Endothelial markers play an important role in the identification and investigation of cells, while their expression displays the heterogeneity in various tissues and organs²⁴. FVIII, CD31, and CD34 are constitutively expressed in vascular endothelial cells and accepted to be the pan-endothelial markers²⁵, so they were detected in this study. As shown in the results, the positive-cell percentages of FVIII CD34 and CD31 had no significant differences both in the primary cell culture and separated MVECs. Interestingly, the fluorescence intensity of FVIII⁺ cells was higher in MVECs than in the primary cell culture. Effects of magnetic field on endothelial functions had been documented^{19,20}, and the result suggests that the expression of FVIII may be increased by magnetic separation. Whether its expression would recover after several passages remains further studies.

Conclusion

In conclusion, highly pure rat intestinal mucosal MVECs can be isolated from the primary cell culture using magnetic beads coated with an anti-CD31 antibody, while their functions may be influenced by magnetic separation.

Declarations

Acknowledgment

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Author contributions

T.Z. conceived the work, Y.W., Z.W., G.H., and T.Z. designed the experiments, Y.W. wrote the article and prepared the figures; Y.W. and G.H. interpreted the data; All authors edited and commented on the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Magnetic separation of CD31+ cells from rat primary intestinal mucosal cell culture. A: Single-cell sample from rat jejunal mucosa; B: Sample cells and immunomagnetic beads conjugated CD31 antibodies; C/D: Separated CD31+ cells binding with beads at low (C) and high (D) magnification. Scale: 100 μm (A, B, and C), 50 μm (D).

Figure 2

Confluent rat intestinal mucosal cell culture before (A) and after (B) magnetic separation. Scale: 200 μ m.

Figure 3

Growth curve of rat intestinal mucosal microvascular endothelial cells at different passages using the MTT method (A) and the number counting method (B).

Figure 4

Immunofluorescence staining of three markers in rat intestinal mucosal cell culture before and after magnetic separation. Scale: 50 μ m.

Figure 5

Percentage and mean fluorescence intensity of positive cells by immunofluorescence staining before and after magnetic separation. * $P \leq 0.05$; ** $P \leq 0.01$.