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Cell rolling column in purification and differentiation analysis of stem cells

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ABSTRACT

Various types of stem cell have been studied as potential cell sources in regenerative medicine. In particular, the autologous stem cells such as mesenchymal stem cells are being widely examined because they can be easily harvested from the patient. For regenerative medicine to become a safe and common practice, stem cells that exist together with various other kinds of cells in the organs must be isolated and purified without any loss of cell functions such as cytokine production or cell differentiation ability. Here, we briefly review the cell separation methods and introduce our original cell separation method based on the cell rolling phenomenon. Mesenchymal stem cells (MSCs) were conventionally isolated by using the adherent property of bone marrow cells onto a plastic culture dish, but they were considered as phenotypically and functionally heterogeneous. We developed a ligand-immobilized surface for separating a subpopulation of adherent cells derived from bone marrow and successfully isolated two cell populations with high differentiation ability for osteoblasts.

1. Introduction

Regenerative medicine is the process of reproducing the lost function of the tissue/organs due to age, disease, damage, or congenital defects by artificially using the intrinsic healing capacity of living organs or cells with a controlled appropriate microenvironment for the stem cells. Regenerative medicine presents potentially attractive alternatives to the use of artificial organs or organ transplantation. Recently, autologous cell transplantation has been widely carried out clinically because of the safety of the procedure. As the cell source, autologous stem cells such as mesenchymal stem cells have been widely examined because they can be easily harvested from the patient. For regenerative medicine to become a safe and common practice, stem cells that exist together with various other kinds of cells in the organs must be isolated and purified without any loss of cell functions such as cytokine production or cell differentiation ability. In the case of embryonic stem (ES) cells and the induced pluripotent stem (iPS) cells, which recently have been receiving increasing interest, it is possible to get a sufficient number of cells from a single cell colony. However, since various cell types or stages appear during the cell differentiation procedure, it is necessary to check the individual cell features and to prepare as uniform a cell population as possible. The development of an effective and safe cell isolation system is thus very important for improving regenerative medicine. In the present study, we focused on the separation methods of stem cells, and we describe our recent results in using a cell rolling column for stem cell separation.

2. *Stem cells in regenerative medicine*

Rapid advancements in stem-cell research have greatly affected regenerative medicine and cell transplant therapy. Human ES cell establishment by Thomson in 1998 [1], information about the separation and differentiation capacity of mesenchymal stem cells (MSCs) reported by Pittenger in 1999 [2], the report on the plasticity of MSCs by Verfaillie in 2002 [3], and the establishment of induced pluripotent stem cells (iPS) by Yamanaka in 2007 [4] greatly increased the possibility of cell sources for use in regenerative medicine. However, using these kinds of stem cells for regenerative medicine as a common strategy in clinical stage will require more research. Homogeneity of the cell type and differentiation stage and protection from infectious issue must be taken into consideration, for the safe and effective practice of regenerative medicine. Scheme 1 shows some of the stem cell sources. Among these cells, autologous transplantation of MSCs derived from bone marrow or adipose tissue has been carried out clinically, due to the lower risks of this procedure and the easier isolation of MSCs.

2.1. *Embryonic stem cells (ES cells)*

Thomson et al. reported the establishment of human ES cells in 1998 [1]. ES cells were derived from the inner cell mass of blastocysts; they are known to differentiate into nerve cells [5], blood cells [6], cardiomyocytes [7,8], and other cells. The pluripotency

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Undifferentiated Stage	Cells	Advantages	Problems
Highly undifferentiated	ES	Pluripotent	Teratoma formation Ethical issue
	iPS	Pluripotent Autologous	Teratoma formation
	MSCs	Autologous	Heterogenicity Limited differentiation lineage
Less undifferentiated	HSP EPC	Autologous	Limited differentiation lineage

Scheme 1. Cell sources for regenerative medicine.

of the ES cells is a valuable feature, but at the same time they may differentiate into cells of unexpected types and may form teratomas after transplantation. Therefore, it is better to prepare target cells by inducing ES cell differentiation and to purify the cells thoroughly in vitro. In addition, since ES cell establishment is accompanied by the death of the fertilized human embryo, the use of this practice raises ethical issues.

2.2. Induced pluripotent stem cells (iPS cells)

Yamanaka et al. originally reported that human fibroblasts can be reprogrammed by transformation with four genes (Oct3/4, Sox2, Klf4, c-Myc), giving ES cell-like immature cells [4]. These pluripotent cells are called induced pluripotent stem cells (iPS cells) and have been studied worldwide. Recently, iPS preparation procedures that do not use the Myc gene, which is one of the oncogenes, and that use viral vectors for gene transfer have been reported [9]. Regarding cell transplantation, iPS cells possess the same teratoma formation issue as ES cells have, but unlike the case with ES cells, autologous iPS cells can be prepared from the patient's cells.

2.3. Mesenchymal stem cells (MSCs)

In 1999, Pittenger et al. reported the existence of MSCs that can differentiate into osteoblasts, chondrocytes, and adipocytes, in the adherent cell fractions of the bone marrow cells [2]. This research team's purification method is very easy and then even now is considered the gold standard for MSC preparation. However, only a few percent of the adherent cell fractions possess the differentiation capacity of the stem cells. Due to the plasticity of MSCs [2,10], they may differentiate into the other types of cells. Since MSCs do not form teratoma and can be easily isolated from the patient, the autologous cell transplantation have been studied widely in preclinical and clinical stages.

2.4. Hematopoietic stem cells (HSCs) and endothelial progenitor cell (EPCs)

In 1960, hematologist Ernest McCulloch and physicist James Till reported the existence of hematopoietic stem cells (HSCs) [11]. After that, Nakauchi's group and Ziegler's group identified mouse and human HSCs, respectively [12,13]. Because the surface marker of the HSC has been strictly identified, it is possible to purify them using a fluorescence activated cell sorter (FACS). There are two subpopulations of HSCs, called short-term HSCs and long-term HSCs. Long-term HSCs function for a long period of time as stem cells, while short-term HSCs do not. The separation of these two populations based on the cell surface marker is being studied [14,15]. In addition, in 1997, endothelial progenitor cells (EPCs) were discovered by Asahara et al. and identified as the precursor cells for forming blood vessels [16,17]. These less undifferentiated stem cells do not form teratomas.

3. Stem cell separation

One of the biggest issues in using stem cells is the establishing the technique for purifying and maintaining their undifferentiated state of stem cells. For highly efficient cell transplantation therapy, a stem cell population with high therapeutic efficacy must be prepared. There are two processes in stem cell separation. One is cell isolation from tissues, organs, blood, or bone marrow. The former requires that the bulk tissue be treated mechanically (homogenization) or enzymatically (digestion) to provide the cell suspension. These treatments can greatly affect the stem cell viability and functions. In contrast, MSCs or HSCs can be easily isolated from the peripheral blood or the bone marrow using a syringe. The other mode is to purify stem cells from cells with different phenotypes or at various differentiation stages.

Since bone marrow-derived MSCs are a useful source for cell transplantation, various methods for purifying the MSCs with expected functions have been developed, as described in Scheme 2. In general, the cell separation is based on the physical properties (size or density) or biological properties (surface markers) of the cells. Some methods allow separation of the stem cells in a continuous manner but others do not. We call the process performed by the latter type of system digital type cell separation. One example of digital type cell separation is the magnetic activated cell sorter system (MACS). The MACS system can divide the cells based on the marker molecule into two populations, the positive and negative populations. In contrast, continuous-type cell separation is the technique with which the weakly positive cells can be separated from the strongly positive cells in a continuous manner. The continuous-type cell separation method is not widely used. Dipole magnet flow fraction (DMFF) can separate the cells depending on how many magnetic beads are bound onto the cell surface, and then the continuous cell separation can be achieved. Since the stem cell surface marker expression is changing continuously depending on the differentiation stages, it is of prime importance to purify the stem cells in a continuous manner.

3.1. Cell sorting by the electric field

The FACS, which is also one of the continuous methods, identifies the target cells carrying the fluorescent probe-labeled antibody against the specific cell surface marker, and can sort the cells using the electric field. It is possible but complicated to sort the cells by using plural cell surface markers. Since the fluorescence intensity of each cell can be measured and picking up the cells with specified intensity by gating is possible, FACS is a very powerful tool for basic stem-cell research. Another method for continuous cell separation, based on detecting the cellular electronic features, has also been reported [18–20]. With this method, since the cells do not have to be labeled by an antibody or a chemical, the purified cells contain no contaminants.

3.2. Cell separation by the magnetic fields

In the MACS system, the magnetic bead-labeled antibody binds only to the target cell surfaces, and the labeled cells can be separated from the unlabeled cells [21–23]. The major advantage of the MACS system is its simple procedure. The cell sample is just mixed with the magnetic bead-labeled antibody against the target cell surface marker, and the target cells carrying the magnetic beads are easily separated from the negative cells using a magnet.

Cell property	Force field	System	Type
Physical Property	Gravity	Density gradient Ultracentrifugation	Digital
	Adhesion	Common Culture	Digital
Biological Property	Electronic	DEP (Dielectrophoresis)	Continuous
		FACS (Fluorescence Activated Cell Sorter)	Digital (Gating)
	Magnetic	DMFF (Dipole Magnet Flow Fraction)	Continuous
		MACS (Magnetic Activated Cell Sorter)	Digital
	Hydrodynamic	Cell Rolling Column	Continuous
		Cell Adhesion Membrane	Digital

Scheme 2. Cell separation systems.

To treat a large number of cells is also easy. Therefore, devices that can be used clinically have already been developed. Using this system, an effect of T cell removal on the graft versus host disease (GVHD) has been reported [24]. Recently, the continuous cell separation using the magnetic bead method under a continuously changing magnetic field has also been reported [25].

3.3. Separation using hydrodynamic force

Cells can be separated by hydrodynamic shear stress in a microfluidic system that includes interaction with the cells. Kato et al. reported the lineage-CD34 cell separation using polyethylene terephtalate filters [26]. Nagrath and Sequist succeeded in catching circulating cancer cells in the bloodstream by using a microchannel with 100-μm pillars modified with the specific antibody in the Circulating Tumor Cells (CTC)-chip diagnosis method [27]. An adhesion-based cell separation chip was also reported by Chang et al. [28]. In related work, our group has been studying a novel system for continuous separation of stem cells with different surface marker expression levels by using the dynamic interaction of the cell surface and a solid surface. In this system, the cells, which are weakly adhering to a solid surface via multiple specific interactions between cell surface marker molecules and the corresponding antibody, received the hydrodynamic force and rolled on the solid surface. Because the rolling speed is determined by the number of interactions as shown in Fig. 1, this system works as a continuous-type cell separation column. HSCs and EPCs are well defined and are useful for cell purification in the FACS system. In contrast, marker molecules for the MSCs whose undifferentiation tendency is much higher than that of HSCs and EPCs are still unclear, and the expression levels of the marker molecules seem to change as they differentiate. Studies have shown that the CD34 level of MSCs or HSCs is not stable [29,30].

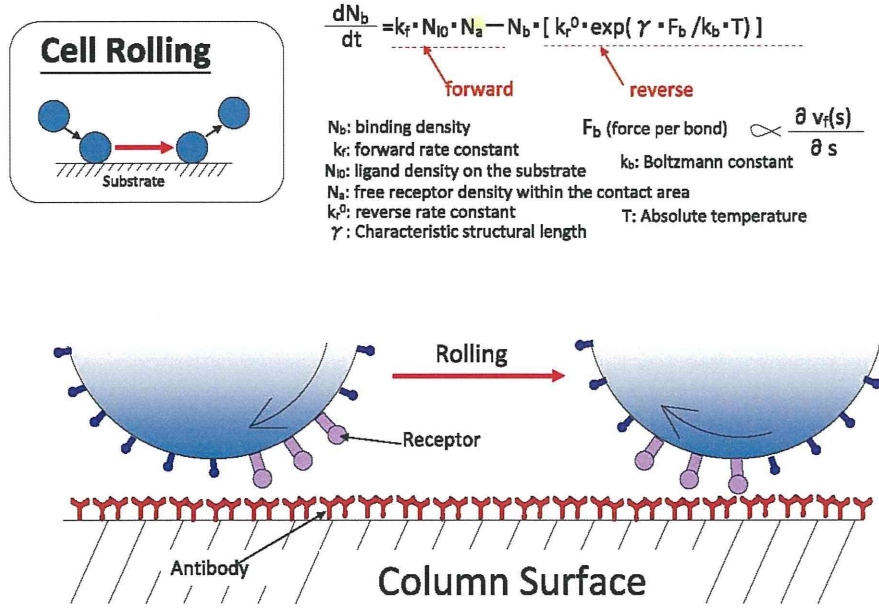

Fig. 1. Cell rolling on the antibody-immobilized solid surface.

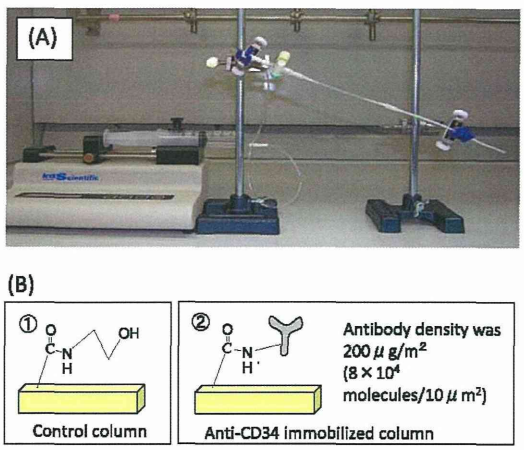

Fig. 2. (A) Appearance of the cell rolling column and (B) surface chemistry of the column lumen.

4. Development of a cell rolling column

We have recently developed an antibody-immobilized column that can separate MSCs on the basis of the CD34 marker expression level [31,32]. The separation mechanism is based on dynamic interaction between the cell surface marker (CD34) and an immobilized antibody and is known as cell rolling [33]. The rolling velocity is regulated by the ligand or cell surface receptor density [34–38]. Silicone tubes with 0.5-mm inner diameter were used as a substrate for the antibody-immobilized column. Graft polymerization of acrylic acid onto the silicone tube surface was conducted as follows [39,40]. The tube was treated with ozone gas for 4 h, dipped in 10% acrylic acid/methanol solution, and incubated at 60 °C. After 4 h, the tube was washed with water. To immobilize anti-CD34 antibody on the tube surface, we preactivated the poly(acrylic acid)-grafted tube with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC), filled with the anti-mouse CD34 rat IgG antibody solution at a concentration of 10 μg/ml, and incubated the tube at 37 °C for 15 h. The tube was washed with phosphate buffered saline (PBS), treated with 1 mM 2-aminoethanol solution for 1 h, and preserved at 4 °C until experimental use. The column length was 10 cm, and the tilt angle was 20° (Fig. 2). A total of 2×10^4 crude MSCs in 10 μL of PBS was injected into the column. The column was flushed with PBS at a flow rate of 50 μL/min until the flow volume reached 250 μL, and at 600 μL/min thereafter. The eluted cell suspension was collected from the top of the column, and cell suspensions were fractionated by elution volume (12.5 μL per fraction).

The number and surface marker profile of cells in each fraction were analyzed by the FACS system (Fig. 3) [32]. The figure clearly shows that delayed fraction at the fraction numbers 8–10. We then used FACS to evaluate the surface marker expression of the isolated MSCs on the anti-CD34 antibody-immobilized column. MSCs with a high expression of CD34 and Sca-1 were presented in the retarded fractions, and a continuous change in the marker expression level was also confirmed on FACS, suggesting that the antibody-immobilized column could be useful to isolate MSCs continuously on the basis of their surface marker density.

The osteoblastic differentiation capacity of the MSCs in each fraction was evaluated by gene expression analysis (Type 1 collagen, osteonectin, CBFA1, and osteopontin) and alizarin red S staining. Type 1 collagen and osteonectin are constantly expressed during osteoblastic differentiation [41,42], while CBFA1 is expressed during the process of maturation. In the case of CBFA1, the expression level in fractions 3, 5, and 6 was higher than that in other fractions. After the differentiation induction, the MSCs were stained with alizarin red S solution, which is also shown in Fig. 3. Isolated MSCs in early fractions (fractions 2 and 3) or later fractions (fractions 5–7) were strongly positive. This staining pattern in terms of the fraction number was similar to that of the CBFA1 expression pattern. These results suggest that separated MSCs in early fractions or later fractions had a high potential for osteoblastic differentiation. It has been reported that osteoblastic progenitor cells were enriched in the CD34-positive population from bone marrow [43]. Our results clearly showed that there are two possibilities with regard to their origin. First, the osteoblastic progenitor cells in bone marrow were contaminated in culture dish-adherent cell fractions, and second, a fraction of MSCs differentiated into progenitor cells during the cultivation [44].

In nature, cell rolling is mainly observed in blood vessels as an inflammatory response of leukocytes [33], and its mechanism is derived from temporary interaction between the cell surface and ligands. Our separation technique would principally enable a labeling-free process, and the isolated cells would not be contaminated with fluorescent or magnetic-labeled antibody.

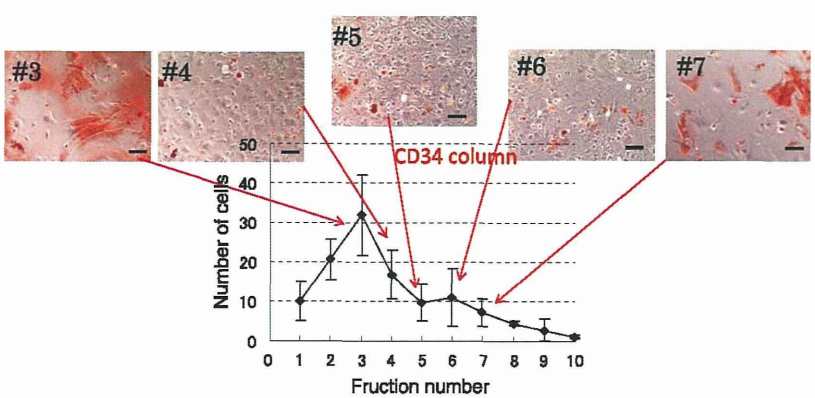

Fig. 3. Osteoblastic differentiation property of isolated MSCs on the CD34 antibody-immobilized column. Scale bar shows 10 μm.

5. Conclusions

An anti-CD34 antibody-immobilized column was developed for separating MSCs based on their surface maker density. We selected the anti-CD34 antibody as the immobilized ligand, and crude MSCs were separated on this column. We succeeded in separating two cell populations with a high ability for osteoblastic differentiation. Not only the cell separation technology but also the other novel technologies, including injectable scaffold, cell sheet technology, and cell tracking technology, will play important roles in the translational research of stem cell-based regenerative medicine in the future.

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Development of cell rolling column modified with betain polymers

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Key: Biomaterials/Cell Rolling/Amphiphilic Polymer

[PREFACE]

Human ES and iPS cell research increased remarkably in recent years since separation of single type cells is a very important issue for these stem cell researches.

An efficient separation system is needed to isolate the specific cell population. The surface marker of the cells has been using for the conventional cell separation. However, there is a problem that in almost cases, the cells have to be labeled with antibody and it would contaminate the cells. In the previous study, our group has focused on the cell rolling and developed the cell separation silicon column and glass column with the antibody immobilized interface through the poly(sulfopropyl betain) brush[1, 2]. However, the polymerization should be performed in the column. In this study, we synthesized an amphiphilic copolymers composed of 2-methacryloyl oxyethyl phosphorylcholine (MPC) as hydrophilic segment and *n*-butyl methacrylate (*n*BMA) as hydrophobic segment (Figure1 (a)) and coated the column surface via hydrophobic interaction. The antibody was immobilized on the surface of micro-chamber that has a vertical crossed flow channel. The cells were injected and settled out, and cell rolling behavior was evaluated.

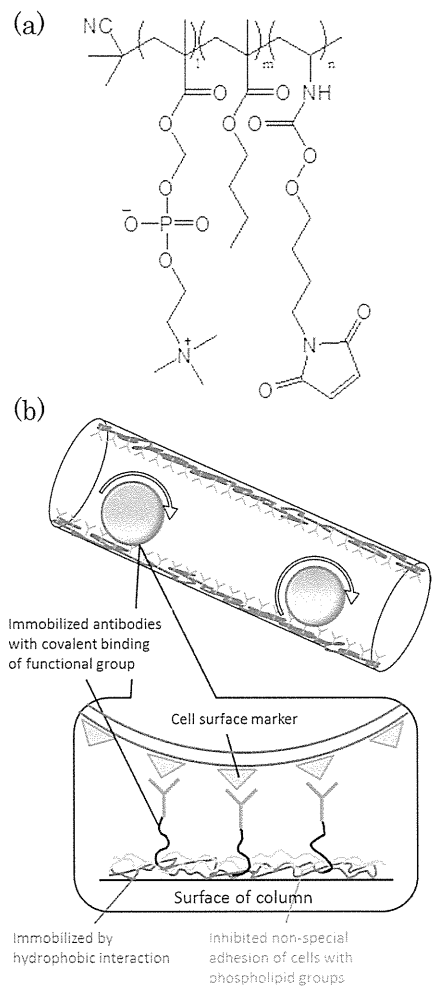

Figure.1 (a) Chemical structure of MPC-*n*BMA-NVA and (b) cell rolling on the polymer coated surface.

[RESEARCH]

The *random*-copolymers were synthesized by free radical polymerization of MPC that has betain group, *n*MBA, and *N*-vinylformamide(*N*VFA) (MPC-*n*BMA-*N*VFA). The poly(*N*VFA) segment was changed to primary amino

group by hydrolysis with hydrochloric acid (MPC-*n*BMA-*N*VA, Table.1) and purified by the dialysis. Poly(ethylene grycol) that has succinimide group and maleimide group was reacted with the copolymer to introduce the maleimide group. The copolymer was dissolved in ethanol and coated on slide glass surface that was cleaned by ozone. The coating was evaluated by X-ray photoelectron spectroscopy and water contact angle measurement. The micro-chamber that has a vertical crossed flow channel was treated with the copolymer and anti-CD34 antibody was immobilized. The suspension of HL60 (CD34 negative) or KG-1a (CD34 positive) was injected and observed by high-speed camera. To increase the interaction of cells on the interface, the micro-chamber was remained to settle out cells on channel surface.

Table.1 Synthesized copolymers.

Sample	MPC:*n*BMA:*N*VA (mol)
NVA 1.0%	30:59:1
NVA 0.5%	30:59.5:0.5
NVA 0.1%	30:59.9:0.1

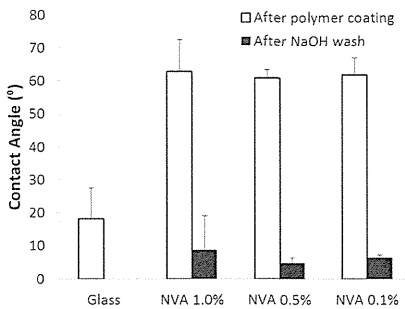

Figure.2 Contact angle analysis of the courted grass surface with MPC-*n*BMA-*N*VA

[RESULTS AND CONCLUSIONS]

The molecular weight of synthesized copolymer (Table.1) was determined by ^{1}H-NMR and GPC. As the result of XPS measurement, the ratio of nitrogen and phosphorus on slide glass surface was increased after the coating. The water contact angle of glass after ozone cleaning was 20°, and the angle was increased to 70° after the polymer coating. When the copolymer coated glass surface was washed with 2.0 N NaOH.aq, the angle was decreased to 10°(Figure2), indicating that the copolymer layer was formed on the glass surface.

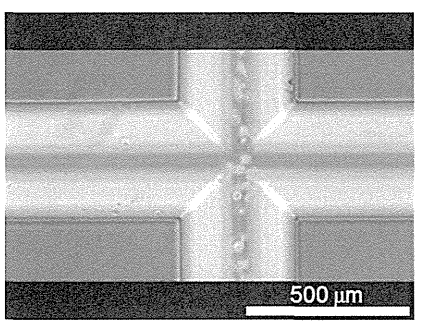

Figure.3 Cross-type micro channel (Volume of cross aria: 12nL、volume of micro channel: 2.9µL)

After the injection of cell suspension into antiCD34 antibody-immobilized micro-chamber, the non-specific adhesion was not observed. The amphiphilic copolymer coating was easy method to modify the glass surface and effectively inhibit the non-specific adhesion.

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Cell-rolling microchip for the detection of circulating-tumor cells

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INTRODUCTION

Circulating tumor cells (CTCs) are existing in the peripheral blood of cancer patient, and relation of CTCs to the metastatic spread of carcinomas has been generally recognized in not only basic research field but also the clinical stage[1]. However, a few CTCs are circulating in the blood flow. Recent study focused on the CTCs detection and characterization by using the microchip technology[2-4]. Antibody-immobilized chip and filter system which could capture the specific cells on the microdevice has been widely investigated for the detection of the rare CTCs. However, non-specific absorption of the other cells has not been suppressed in the detection of CTCs, and its sensitivity is greatly reduced. Here, we have developed the cell-rolling microchip for the specific detection of various cells. In our previous work, the cell-rolling column was developed for the separation of stem cells. This column separated the specific cells by the cell rolling velocity under the media flow. In this study, this mechanisms was applied for the cell detection on the microchip systems. The detection sensitivity would be increased by the evaluation of rolling-velocity because of the continuous interaction between the cell surface and immobilized ligand. To immobilize the antibody, copolymers of poly[2-methacryloyloxyethyl phospho-rylcholine (MPC)-*co*-*n*-butyl methacrylate (*n*BMA)-*co*-*N*-vinylformamide (*N*VF)] (PMBV) were synthesized by random polymerization. Microflow pass (width: 300μm, depth: 100μm) were coated with the polymers, and atni-CD34 antibody were covalently attached by the crosslinker. Cell-rolling velocity of cultured cells on the microchip system was evaluated.

EXPERIMENTAL

Polymer synthesis and preparation of microchip Amphiphilic phospholipid polyjmer (PMBV) with MPC, nBMA and NVF was synthesized by radial polymerization of corresponding monomers using α,α'-azobiisobutyronitrile (AIBN) as an initiator. The monomers and initiator were dissolved in ethanol, and the mixture was stirred at 60°C for 6h. After the reaction, the polymers were precipitated twice by hexane and diethyl ether. Composition and molecular weight of the two polymers were determined by 1H-NMR (Geminn 2000/300; Varian Inc., CA, USA), and GPC (Shodex SB804-HQ; Showa Denko K.K., Tokyo, JAPAN) in mixed solvent (EtOH:Pure water =0.7:0.3, included 10mM LiBr), respectively. The MPC/nBMA/NVF composition of PMBV40 and PMBV30 were 0.1/0.5/0.1, and 0.3/0.6/0.1, respectively. To produce the amino group in the polymer, the polymers were hydrolyzed with 60mL of 2N HCl. After the neutralization, the polymers were purified with the dialysis tube (Figure 1).

Microchip for the detection of cell-rolling velocity was manufactured by Institute of Microchemical Technology Co., Ltd., (Kanagawa, JAPAN). The microchip channel pattern was specifically designed for the evaluation of cell-rolling velocity (Figure 2). Microchip channel was coated with the copolymers, and the amino groups of the polymers were activated with the NHS-PEG-Maleimide crosslinker (Thermo Scientific, Hudson, NH, USA). Reduced antiCD34 antibody was added into the channel and then the antibody was covalently conjugated on the channel through the activated copolymers.

Evaluation of cell-rolling velocity on the microchip The microchip was connected to the Microfluidics Flow Control System (MFCS; Fluigent, Paris, FRANCE) to strictly control the media flow in the channel. The KG-1a and HL-60 cells were used as the model cells that cells were CD34 positive and negative cells, respectively. Cell suspension was circulated in the sample channel, and the cells were injected into the detection channel by the change of media flow direction. Rolling velocity were monitored on the CCD camera, and the velocity were analyzed on the personal computer.

RESULTS and DISCUSSION

Molecular weight (Mn) and the molecular weight distribution (Mw/Mn) of the synthesized polymer were about 3-6 × 10^{-4} and 1.1-1.3, respectively. When the glass chip was incubated with the polymer solution, the contact angle was largely changed, and the polymer-derived phosphate and nitrogen signal were observed on the X-ray photoelectron spectroscopy. These results indicated that the polymers were stably absorbed on the grass surface by the courting. The conjugation of the antibody could be traced by the radio-isotope experiments. The glass-made microchip channel was courted by this protocol, and the cell-rolling velocity was evaluated.

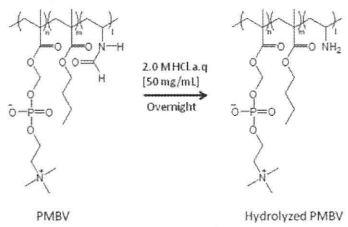

Figure 1 Structure of PMBV and hydrolyzation.

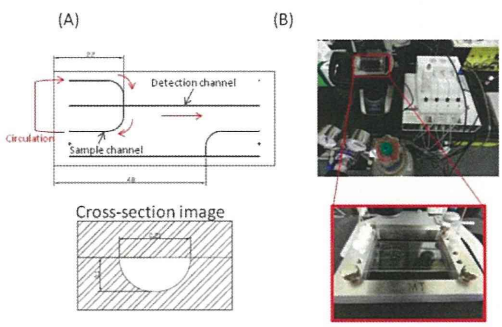

Figure 2 (A) Design of microchip for CTCs detection and (B) the chip and media-flow control system.

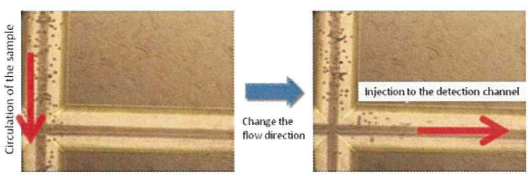

Figure 3 Detection system of cell-rolling velocity on the CTCs microchip channel.

KG-1a and HL-60 cells were used as CD34 positive and negative cells for the model system of CTCs detection, respectively. After the injection of the cell suspension, and the cells were stably circulated by the media flow without any non-specific absorption on the surface (Figure 3). When we used the optimized condition at 0.4 μl/min of media flow, the rolling velocity of KG-1a (CD34 positive) cells on the chip system was detected as about 40 μm/sec. On the other hands, the velocity of the negative cells were about 55 μm/sec. We observed

the significant difference between these rolling velocities. When the media flow was increased, the significant difference of the rolling-velocity was not indicated, and the cell-moving speed was almost same. Therefore, the significant difference was derived from the specific interaction of the surface maker and immobilized ligand.

CONCLUSION

Here, we successfully discriminate the cell type on the microchip by the cell-rolling velocity without any non-specific absorption. This chip system would apply for the CTCs detection system based on the cell-rolling mechanisms.

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