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(Degree)

博士 (医学)

(Date of Degree)

2007-07-11

(Date of Publication)

2013-04-08

(Resource Type)

doctoral thesis

(Report Number)

乙2954

(URL)

<https://hdl.handle.net/20.500.14094/D2002954>

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Conversion of Cultured Monocytes/Macrophages into
Endothelial-like Cells through Direct Contact
with Endothelial Cells

血管内皮細胞との細胞接触による

培養単球／マクロファージの血管内皮様細胞への転換

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Key words: Endothelial cell, peripheral blood monocyte, co-culture, differentiation

Abstract

When culturing human umbilical vein endothelial cells in a culture medium containing 4% human serum albumin, it was possible to maintain the epithelioid morphology and function for several months without subculturing. When co-culturing endothelial cells and labeled monocytes/macrophages (Mo/ Φ) that were collected from peripheral blood and allowed to engulf fluorescent latex beads, some Mo/ Φ changed their shapes and became epithelioid cells that were indistinguishable from vascular endothelial cells. This transformation started within several hours of co-culturing. At 7 days after the start of co-culturing, more than half of the labeled cells were identified as endothelial-like cells morphologically. Furthermore, morphologically altered Mo/ Φ did not express Mo/ Φ -specific antigens, i.e., the MHC Class II molecule and CD68, but expressed VE cadherin and vWF, which are specific antigens for endothelial cells, and labeled cells that changed into endothelial-like cells no longer engulfed fluorescent latex beads. This strongly suggests that peripheral blood monocytes differentiate into endothelial-like cells.

1. Introduction

The half-life of blood monocytes produced in the bone marrow is believed to be about 17 hours, and these monocytes migrate outside vessels and then differentiate into macrophages, osteoclasts, and other phagocytes. Monocytes migrate outside vessels via interactions such as endothelial adhesion. Mo/ Φ and endothelial cells share many similarities in terms of function and antigenicity: 1) specific phagocytosis of denatured LDL, 2) non-specific phagocytosis [1,2,3], 3) expression of blood coagulation tissue factors [4,5], 4) expression of the MHC Class II molecule [6,7], and 5) expression of ulex europaeus lectin-1 [8,9]. Moreover, because both endothelial and blood cells differentiate from the blood island originating from the mesoderm during the prenatal period, they are thought to be related cells.

In recent years, studies have documented that peripheral mononuclear cells contain a subset of vascular endothelial progenitor cells, and that these contribute to neovascularization by becoming embedded in an endothelial cell sheet [10,11]. Moreover, *in vitro* experiments demonstrated that human peripheral blood monocytes have the potential to express specific endothelial antigen under growth factor stimulation [12].

When aortic endothelial cells are cultured for a long period, monocyte and macrophage-like cells appear on a cultured endothelial cell sheet, and they themselves multiply [13]. We have confirmed this by culturing umbilical vein endothelial cells for a long period. This phenomenon can be explained as follows: monocytes and macrophages that strongly adhere to endothelial cells begin to multiply when co-cultured with endothelial cells. However, we have found that macrophage-like cells do not appear locally, but they continue to multiply over time in a dispersive manner from a single-layer cobblestone-like endothelial cell sheet. Therefore, it appears reasonable to assume that the phenomenon is due to the conversion of endothelial-like cells into macrophage-like cells.

Taking into account the above results, the following hypothesis could be formulated: when Mo/ Φ come into contact with endothelial cells, at least some Mo/ Φ change into cells that are morphologically and functionally indistinguishable from endothelial cells. Furthermore, Mo/ Φ that have morphologically become endothelial-like cells can revert back to macrophage-like cells.

Therefore, we co-cultured endothelial cells and human peripheral blood monocytes and used fluorescent tracers to closely examine whether the Mo/ Φ changed into endothelial-like cells morphologically, antigenically, and functionally.

2. Materials and Methods

2.1. Endothelial cell and Mo/ Φ culture solution preparation and dish coating

Endothelial cell culture solution: Endothelial cells were cultured using medium 199 Earle's solution (M-199) containing human serum albumin (albumin). To M-199 (Sigma-Aldrich, Inc. (Sigma)), 4% (final concentration) human albumin (Mitsubishi Wellpharma, 25% blood albumin), 5 $\mu\text{g/mL}$ insulin (Sigma), 100 ng/mL cortisol (Sigma), 100 U/mL penicillin (Sigma), 0.1 mg/mL streptomycin (Sigma), and 2.5 $\mu\text{g/mL}$ amphotericin B (Sigma) were added, and 1M NaOH was used to adjust the pH to 7.4 ± 0.05 . For endothelial cell culture, 20% FBS (Sigma) was also added.

Monocyte culture solution: To M-199, 5 $\mu\text{g/mL}$ insulin, 100 ng/mL cortisol, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2.5 $\mu\text{g/mL}$ amphotericin B, and 20% FBS were added.

Dish coating: For endothelial cell culture, dishes coated with type IV collagen were used. Type IV collagen (Nitta Gelatin Cellmatrix type IV) was diluted ten-fold using 10^{-3} M HCl, and about 0.1 mL/cm² of the diluted solution was placed in each 35-mm plastic dish, 60-mm plastic dish, and 24-well tissue culture plate. Dishes and plates were left to stand at room temperature for about 1 hour. The collagen solution was aspirated, and the dishes and plates were then washed three times using PBS containing Ca²⁺ and Mg²⁺ (PBS+, Sigma). However, Mo/ Φ were cultured using uncoated dishes.

2.2. Collection and culture of human umbilical vein endothelial cells

A cannula was inserted into each end of a human umbilical vein, and, after fixing both ends, Ca²⁺ and Mg²⁺-free PBS (PBS-, Sigma) was used to perfuse the vein to remove as much blood as possible. The vein was then filled with PBS-, and after about 3 hours of incubation at 37°C, the vein was filled with

PBS⁻ containing 0.5 mg/mL (final concentration) of trypsin and 0.2 mg/mL of EDTA and incubated for 20 minutes. The PBS solution containing endothelial cells from the vein was then washed into a test tube using M-199. The test tube was centrifuged at 1000 rpm for 10 minutes, and after discarding the supernatant, the precipitate was obtained. After suspending the precipitate in M-199 and centrifuging the resulting solution, the precipitate was dispersed in the endothelial cell culture solution and cultured in a type IV collagen-coated dish. The culture solution was exchanged every few days.

2.3. Subculturing of endothelial cells

Primary cultured endothelial cells were washed three times using PBS⁻ and incubated for 1-2 hours at 37°C with 0.1 mL/cm² of PBS⁻. Next, PBS⁻ containing 0.5 mg/mL (final concentration) of trypsin and 0.2 mg/mL EDTA was added. After microscopically confirming that the cells were detached, FBS was used to inactivate trypsin, and M-199 was added to recover suspended cells. After centrifugation, the cells were dispersed in the endothelial cell culture medium and incubated using a type IV collagen-coated 24-well tissue culture plate.

2.4. Human peripheral blood monocyte isolation and culture

Human peripheral blood samples were obtained from normal volunteers in their 20's to 50's using heparinized syringes and the mononuclear cell fraction was collected using Histopaque-1077 (Sigma). The resulting mononuclear cells were washed and centrifuged twice using PBS⁻, dispersed in the monocyte culture solution, and placed in a dish. After 72 hours, cells that had not adhered to the dishes were washed and removed using PBS⁺. The adherent monocytes continued to be cultured.

2.5. Phagocytosis of fluorescent beads

As fluorescent beads, 1 μm microspheres (Molecular Probes Inc.) with blue or orange fluorescence were used. To the culture solution of each cell culture dish or plate, about 10^6 beads/mL were added, and cells were incubated at 37°C for 3 hours. The cells were then washed three times using PBS+ and the culture solution was added. The resulting cells were examined under a fluorescence microscope. The number of beads engulfed by cells was counted manually on images.

2.6. Cell nucleus staining

Cell nuclei were stained by diluting Hoechst 33342 (Dojindo Laboratories) using M-199 to 30 μM and adding it to each dish and plate at a volume of one tenth the culture solution. After incubating at 37°C for 15 minutes, the cells were washed twice using PBS+, and the culture solution was added. The resulting cells were examined under a fluorescence microscope.

2.7. Confirmation of cell-specific antigen expression

The expression of Mo/ Φ and endothelial cell-specific antigens was confirmed by immunofluorescence staining. Cells were fixed using 4% paraformaldehyde (Wako Pure Chemical Industries Ltd.) for 30 minutes, and with the exception of those used for MHC Class II molecule detection, they were treated with 1% Triton/PBS+ for 10 minutes followed by primary antibody.

As the primary antibodies for detecting CD68 and MHC Class II molecule, which are Mo/ Φ -specific antigens, anti-MHC Class II molecule monoclonal antibody (910D7, BMA Biomedicals) and anti-CD68 monoclonal antibody (KP1, Ylem S.R.L.) were used. For the detection of VE-cadherin and vWF, which are

endothelial cell-specific antigens, anti-vWF monoclonal antibody (F8/86, DACO) and anti-VE-cadherin monoclonal antibody (BV6, Chemicon International Inc.) were used. Rhodamine-labeled anti-mouse IgG antibody (Chemicon International Inc.) was used as a secondary antibody.

3. Results

3.1. Culture of endothelial cells

Endothelial cells collected from the human umbilical vein were cultured in the endothelial cell culture medium. In the primary culture, cells reached confluency in about 1 week, and a cobblestone-like morphology was observed. The resulting cells were subcultured, and, as was the case with primary culture, cells reached confluency and developed a cobblestone-like morphology. These endothelial cells maintained this appearance for several months without subculturing. After 1 month, these cells still absorbed acetylated LDL and expressed vWF. Furthermore, when cultured using the endothelial cell culture medium containing human albumin, cells multiplied and maintained confluency without growth factors such as VEGF.

When endothelial cells were cultured using culture solution free of albumin, after reaching confluency, cell viability gradually decreased and intercellular gaps increased. Furthermore, some cells acquired a 'sprouting' phenotype. Therefore, cells could not maintain the cobblestone-like morphology for 1 month. Moreover, growth factors were required for multiplying endothelial cells in this culture solution.

3.2. Phagocytosis of labeled beads by endothelial cells

Studies have reported that, under various conditions, non-specific phagocytosis of endothelial cells can be induced. In order to confirm whether

phagocytic activities of endothelial cells were induced under the present culture conditions, fluorescent beads were added to culture medium containing albumin. Most typical confluent endothelial cells did not engulf labeled beads, and only 3-4% of the cells engulfed 1 or 2 beads.

3.3. Mo/ Φ culture and fluorescent labeling

Monocytes that were collected from human peripheral blood and had adhered onto dishes, extended pseudopods and gradually became macrophage-like in morphology.

At about 10 to 14 days after the start of incubation, fluorescent beads were added to the culture medium, and cells were incubated for about 3 hours so that they could engulf beads. More than 95% of the adherent Mo/ Φ cultured for 10-14 days engulfed at least 3 to 4 beads. Furthermore, we confirmed that almost all adherent cells expressed CD68 and MHC Class II molecules.

3.4. Changes in Mo/ Φ morphology through co-culturing of endothelial cells and labeled Mo/ Φ

The culture solution of Mo/ Φ labeled with fluorescent latex beads was replaced with PBS-, and cells were incubated for 2-3 hours and then suspended. Adherent cells were also suspended by pipetting and then recovered. After centrifuging the recovered cells at 1000 rpm for 10 minutes, the resulting cells were suspended in the endothelial cell culture solution and added over confluent endothelial cells. Labeled Mo/ Φ could be seen under a fluorescent microscope based on the presence of fluorescent tracers (Figure 1). After the start of co-culturing, morphological changes of labeled Mo/ Φ were observed. Two to three hours after the start of incubation, some labeled Mo/ Φ became embedded into endothelial cell gaps and flattened. These cells kept increasing in size and gradually became endothelial-like cells (Figure 2). After

24 hours, even more labeled cells had become endothelial-like cells. This morphological change continued to be seen with time. The frequency of the morphological change varied depending on samples, but with some samples, more than half the labeled cells had become endothelial-like cells 7 days after the start of co-culturing (Figure 3).

However, some labeled Mo/ Φ continued to remain spherical and macrophage-like for 14 days without becoming endothelial-like cells. When the endothelial cell sheet was physically detached, some labeled Mo/ Φ were under the endothelial cells and had directly adhered to the dish with a macrophage-like morphology.

After Mo/ Φ engulfed 1 μ m latex beads with blue fluorescence, the MHC Class II molecules of Mo/ Φ were labeled using anti-MHC Class II molecule monoclonal antibodies as primary and Rhodamine-labeled anti-IgG antibodies as secondary antibodies. The Mo/ Φ double-labeled with fluorescent beads and anti-MHC Class II antibodies were co-cultured with endothelial cells. The Mo/ Φ embedded into endothelial cells and developed an endothelial cell-like morphology (Figure 4).

3.5. Mo/ Φ cultured alone

At the same time as endothelial cells and labeled Mo/ Φ were co-cultured, labeled Mo/ Φ were cultured on their own. Labeled Mo/ Φ adhered to the dish and extended pseudopods. These cells morphologically remained monocytes or phagocytic cells.

3.6. Phagocytosis of fluorescent beads by co-cultured endothelial cells and Mo/ Φ

At 3 and 7 days after the start of co-culture, fluorescent beads of another color were used to identify phagocytosis.

At 3 and 7 days after the start of co-culture of the labeled Mo/ Φ with endothelial cells, morphologically monocytic cells engulfed beads at a comparable level to Mo/ Φ labeling. However, labeled endothelial-like cells did not engulf beads (Figure 5). Some cells that changed into macrophage-like cells did not engulf beads, probably because they were under endothelial cells.

The endothelial cells that were co-cultured with Mo/ Φ could be differentiated because they did not engulf the beads used for Mo/ Φ labeling. Most of these endothelial cells did not engulf beads of another fluorescence color that were added. Therefore, when co-cultured with Mo/ Φ , the phagocytic activity of endothelial cells was not induced.

Furthermore, when labeled Mo/ Φ were cultured on their own, approximately 10% did not engulf beads. When compared to labeling, the number of engulfed beads was slightly lower, but almost all Mo/ Φ maintained their phagocytic activity.

3.7. Cell nucleus staining

In order to confirm that the morphological change from labeled Mo/ Φ to endothelial-like cells was not due to the cellular fusion between labeled Mo/ Φ and endothelial cells, cell nuclei were stained using Hoechst 33342. Endothelial cells without fluorescent beads and cells with fluorescent beads that appeared to have changed from Mo/ Φ possessed only one nucleus, and there were no marked differences in the fluorescence intensity of Hoechst 33342 per cell (Figure 6).

8. Changes in the antigenicity of Mo/ Φ induced by co-culturing

At 3 and 7 days after the start of co-culturing, antigens expressed by Mo/ Φ were analyzed. The changes in antigenicity of co-cultured Mo/ Φ were investigated in terms of the Mo/ Φ -specific antigens, MHC Class II molecule

and CD68, and the endothelial cell-specific antigens, VE-cadherin and vWF. The same experiment was also conducted using Mo/ Φ that were cultured on their own.

At 3 and 7 days after the start of co-culturing, spherical labeled cells without a morphological change expressed the MHC Class II molecule and CD68. Moreover, labeled cells that had begun to change into endothelial-like cells and became flattened expressed the MHC Class II molecule and CD68. However, labeled cells that changed into endothelial-like cells either did not express the MHC Class II molecule and CD68 or expressed very low levels of these antigens (Figures 7A and 7B). Mo/ Φ that were incubated on their own expressed MHC Class II molecule and CD68. Labeled Mo/ Φ that had not undergone a morphological change did not express vWF. Labeled cells that had undergone an endothelioid morphological change expressed vWF, but the level of expression was weak in some cells. Some labeled cells that were beginning to undergo an endothelioid morphological change expressed a low level of vWF (Figure 7C). More than 95% of the labeled cells that had changed morphologically to endothelial cells expressed vWF. VE-cadherin was expressed by morphologically changed labeled cells and surrounding endothelial cells (Figure 7D). We confirmed that >98% of the morphologically changed labeled cells expressed VE-cadherin. There was no clear expression of VE-cadherin by spherical labeled cells without a morphological change. vWF and VE-cadherin were not expressed by Mo/ Φ that were cultured on their own.

3.9. Incubation of Mo/ Φ using the culture supernatant of endothelial cells

In order to ascertain whether the morphological changes of Mo/ Φ co-cultured with endothelial cells result from factors secreted by endothelial cells, the conditioned medium of the endothelial cells was used to incubate Mo/ Φ .

The culture supernatant of confluent endothelial cells or endothelial cells co-cultured with Mo/ Φ was recovered and filtered using a 0.2 μm membrane filter. Mo/ Φ were cultured using the filtered conditioned medium for about 3 weeks, but the morphology of Mo/ Φ did not change.

4. Discussion

When cultured using growth stimulators such as VEGF and FGF, endothelial cells multiply, and after reaching confluency, growth transiently stops due to contact inhibition. However, after a certain period of time, cells that morphologically resemble macrophages, so-called sprouting cells, appear. Moreover, endothelial cells overlap and begin to grow on top of each other, thus losing the epithelioid morphology of endothelial cells [13,14]. Furthermore, in our experiments, when growth factors were added, endothelial cells resembled phagocytic cells in their engulfing of 1- μm latex beads and labeling with phagocyte-labeling dyes such as PKH26 (data not shown). In the present study, it was necessary to maintain the epithelioid morphology of endothelial cells for a long period of time and avoid inducing non-specific phagocytosis. Therefore, techniques to culture endothelial cells in the absence of growth factors were investigated. The results showed that this could be achieved by adding albumin (final concentration: 4%) to a culture medium.

Using a culture medium with albumin, endothelial cells reached confluency without growth factors. After reaching confluency, macrophage-like cells rarely appeared, and cell stratification barely occurred. This monolayer morphology was maintained for a few months, and the cells absorbed acetylated LDL, expressed vWF, and rarely acquired non-specific phagocytosis, thus suggesting that cultured endothelial cells maintained their morphology and function. Furthermore, even when these cultured endothelial cells were subcultured several times, cell growth was maintained, cellular conditions were stable, and the monolayer morphology was maintained.

Albumin is a protein that accounts for about 4-5% of human plasma proteins, and it is the most common solute in plasma. Endothelial cells exist under a unique physiological environment because they are in constant contact with blood and are affected by blood flow. By adding albumin to a culture solution at a comparable level to that in plasma, endothelial cell growth, morphology, and function could be favorably maintained. In terms of albumin function, maintenance of the colloid osmotic pressure of plasma, transportation of lipids and certain hormones, and antioxidation have been reported. At this point in time, however, it is not clear whether these functions are at work or other functions are involved.

After 10 to 14 days cultivation of the mononuclear cell fraction which was collected from peripheral blood using Histopaque-1077, almost all cultivated cells expressed CD68 and MHC Class II antigen. The majority of cultivated cells showed a macrophage-like morphology, but some cells still maintained the monocytic morphology. These cultivated monocytes/macrophages were labeled with fluorescence latex beads. Labeled Mo/ Φ and endothelial cells cultured under the present conditions were co-cultured. After coming into contact with endothelial cells, many labeled Mo/ Φ became morphologically indistinguishable from endothelial cells.

The morphological transformation of co-cultured Mo/ Φ was seen immediately after the start of co-culturing, and at 2-3 hours after initiating co-culturing, labeled cells that were morphologically indistinguishable from endothelial cells were beginning to be observed. This morphological change continued to be seen, and the number of such transformed cells increased with time. In some cases, at least $\geq 50\%$ of labeled cells became endothelial-like cells within 7 days.

Furthermore, labeled cells that were morphologically indistinguishable from endothelial cells did not express Mo/ Φ antigens such as CD68 and the MHC Class II molecule, but $>95\%$ of the morphologically altered cells expressed endothelial cell antigens such as vWF and VE-cadherin. As shown in

Figure 7, the changes in antigen expression accompanied the morphological changes in Mo/ Φ . Cells that were in the process of undergoing morphological transformation still expressed CD68 and the MHC Class II molecule, which are specific to Mo/ Φ , or expressed vWF, an endothelial cell-specific antigen.

CD68 is a lysosome membrane protein specific to Mo/ Φ [15]. The MHC Class II molecule is a cell membrane protein specific to antigen-presenting cells like Mo/ Φ . In some cases, this MHC Class II molecule is reportedly expressed on vascular endothelial cells. However, MHC Class II molecules are not expressed unless stimulated by cytokines such as IL-1 and TNF- α on cultured human umbilical vascular endothelial cells [6,7]. In the present experiments, cultured endothelial cells did not express these antigens. Of the co-cultured labeled cells, those that were morphologically endothelial-like did not express these antigens, but cells that remained morphologically monocytes or those that had not become completely endothelial-like cells expressed these antigens.

VE-cadherin is involved with homophilic binding of endothelial cells, and like vWF, it is a marker specific to endothelial cells [16,17]. It was clearly seen in the borderline area between endothelial cells and labeled cells that had morphologically changed into endothelial-like cells, thus confirming homophilic bonds among these cells. Like VE-cadherin, vWF was expressed by endothelial and labeled cells that had morphologically changed to endothelial-like cells. Furthermore, during the process of endothelioid changes, labeled cells that had not completely changed into endothelial cells expressed vWF. Hence, vWF is thought to be synthesized in cells from the early stage of morphological changes.

When nuclei of co-cultured cells were stained using Hoechst 33342, like surrounding endothelial cells, labeled cells only had a single nucleus, and the two cell types showed no difference in fluorescence intensity. This suggests that the labeled cells with an endothelioid morphology originated from single cells, rather than through the cellular fusion of endothelial and labeled cells.

Phagocytosis was assessed using fluorescent latex beads of a different color

from those used for Mo/ Φ labeling after the start of co-culturing. Labeled cells that morphologically remained monocytes and were located on top of endothelial cells engulfed fluorescent latex beads and retained non-specific phagocytic activities. Almost all endothelial and labeled cells that had changed morphologically to endothelial cells did not engulf beads; therefore, non-specific phagocytosis was absent or very weak. This finding suggests that it is highly unlikely that endothelial cells nonspecifically engulfed free latex beads that were for some reason released by labeled cells or residual latex beads from the start of co-culturing. Furthermore, the labeled Mo/ Φ with anti-MHC Class II antibody that were co-cultured with endothelial cells became morphologically indistinguishable from endothelial cells.

Therefore, we confirmed that when co-cultured with endothelial cells, Mo/ Φ morphologically transformed into endothelial-like cells, and the antigenicity of these cells also changed to resemble endothelial-like cells. Moreover, because these cells did not engulf fluorescent latex beads, Mo/ Φ appeared to have lost their natural phagocytic activities and to possess altered functions. This suggests that Mo/ Φ may differentiate into endothelial cells.

Mo/ Φ that were co-cultured with endothelial cells gradually became flattened, and these cells kept increasing in size. Due to this increase in the surface area, each individual concentration of MHC Class II molecules/cell decreased. With that decrease, the fluorescence intensity of the labeled MHC Class II molecules decreased. Furthermore, the MHC Class II molecules of cell membranes underwent metabolic turnover, and the newly formed MHC Class II molecules replaced the labeled MHC Class II molecules, and thus, the fluorescence intensity was rapidly reduced. Therefore, it was difficult to use the MHC Class II molecule as a tracer. For that reason, we used latex beads as a common tracer.

In human peripheral blood, vascular endothelial progenitor cells originating from the bone marrow are believed to exist. Therefore, studies have been conducted to ascertain the existence of peripheral endothelial progenitor

cells that were obtained from non-adherent mononuclear cells by means of the stem cell marker CD34. When CD34⁺ mononuclear cells were cultured *in vitro* with various growth factors such as VEGF, the expression of endothelial cell-specific antigens such as vWF, VE-cadherin, Tie-2, and KDR/Flk-1, was observed [10,11]. However, mononuclear cells expressing CD34 only account for 0.2% [18], and vascular endothelial progenitor cells in the peripheral blood alone would be quantitatively insufficient for neovascularization. Therefore, as candidate vascular endothelial progenitor cells, CD34⁻ and CD14⁺ monocytes were collected from peripheral blood to investigate differentiation into endothelial cells. Studies have documented the expression of endothelial cell antigens by CD34⁻ and CD14⁺ monocytes when culturing these cells in a culture medium containing angiogenic growth factors such as VEGF, bFGF, and IGF. However, monocyte- and macrophage-specific antigens were also expressed, and as a result, it is difficult to assume that these mononuclear cells differentiated into endothelial cells [12,19,20]. In the study of Walenta et al., monocytes collected from human peripheral blood were cultured without sorting, and endothelial cell antigen expression was confirmed. However, only a few percent expressed endothelial cell antigens after 10 days [18].

In the present study, Mo/Φ were labeled and then co-cultured with endothelial cells, and at 7 days after culturing, ≥50% of Mo/Φ had morphologically changed into endothelial-like cells and expressed endothelial cell antigens and monocyte- and macrophage-specific antigens disappeared. Our experimental system is based on co-culturing with endothelial cells. Therefore, the following two factors may be involved in the morphological and functional changes of Mo/Φ observed in the culture process: 1) some substances that are secreted from endothelial cells act on Mo/Φ, and 2) direct contact with endothelial cells induces the morphological change of Mo/Φ into endothelial cells.

When filtering the conditioned medium of confluent endothelial cells or that of endothelial cells co-cultured with Mo/Φ with a 0.2 μm membrane to

remove cellular components and then using it to culture Mo/ Φ morphological changes were not seen at all even after 3-4 weeks. Therefore, in order for Mo/ Φ to change into endothelial-like cells, direct interaction between endothelial cells and Mo/ Φ appears necessary.

On direct interaction with endothelial cells, Mo/ Φ become morphologically similar to endothelial cells, and they expressed endothelial cell-specific antigens, while losing Mo/ Φ -specific antigens and non-specific phagocytosis, thus confirming functional changes. The present findings suggest that Mo/ Φ can be converted into endothelial cells through direct contact with endothelial cells.

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Figures legend

Figure 1. A phase-contrast image (A) and a fluorescence image (B) immediately after the start of co-culturing.

A: White spherical monocytes were seen on a layer of cobblestone-like epithelioid vascular endothelial cells.

B: These monocytes had engulfed fluorescent beads, and could therefore be identified as added Mo/ Φ .

Figure 2. Morphological changes of labeled Mo/ Φ co-cultured with vascular endothelial cells.

A: The cell indicated by the arrow extended pseudopods and appeared to be a macrophage (A1). This cell was an Mo/ Φ labeled with fluorescent beads (A2).

B1 and B2: After 2.5 hours, the Mo/ Φ labeled with fluorescent beads became embedded in a gap among vascular endothelial cells. It was slightly larger in size and mostly flattened.

C1 and C2: After 18 hours, the labeled Mo/ Φ had become morphologically indistinguishable from vascular endothelial cells. Eventually, the cell became completely indistinguishable from the surrounding vascular endothelial cells.

Figure 3. Phase-contrast and fluorescent images 7 days after the start of co-culturing.

The fluorescently labeled cells indicated by the arrows in “A” were morphologically monocytic cells on the phase-contrast image. The other labeled cells morphologically became vascular endothelial cells.

Figure 4. Morphological changes of labeled Mo/ Φ with fluorescent beads and anti-MHC Class II antibody co-cultured with vascular endothelial cells (2 days after the start of co-culturing).

The cells indicated by the arrow were fluorescently labeled with latex beads (A)

and anti-MHC Class II antibody (C), thus allowing them to be identified as cells originating from human peripheral blood monocytes (B).

Figure 5. Uptake of fluorescent beads after co-culturing (7 days after the start of co-culturing).

A: The cells indicated by arrows 1-3 were fluorescently labeled, thus allowing them to be identified as cells originating from human peripheral blood monocytes.

B: The cells indicated by arrows 1 were morphologically monocytic cells. The cells indicated by the arrows 2 were Mo/ Φ that had morphologically changed into endothelial cells. The cell indicated by arrow 3 had undergone a slight morphological change.

C: Fluorescent 1 μ m latex beads that had a different color to phagocytosis labeling were used. The Mo/ Φ indicated by arrows 1 maintained phagocytic activities, but cells that had morphologically changed to vascular endothelial cells and cells that had undergone slight morphological changes did not engulf beads.

Figure 6. Nuclear staining of Mo/ Φ that had morphologically changed into endothelial cells.

The labeled cells (A) indicated by the arrows morphologically became vascular endothelial cells (B). The cells possessed only one nucleus and there were no marked differences in intensity (C).

Figure 7. Detection of various specific antigens by immunostaining.

A1 and A2: MHC class II molecule detection. The morphologically monocytic cells with blue fluorescence indicated by arrows 1 were stained for the MHC class II molecule, but the morphologically vascular endothelial cell with blue fluorescence indicated by arrow 2 was not stained for the MHC class II

molecule. The cell with blue fluorescence that began to be changed into a vascular endothelial-like cell indicated by arrow 3 was stained weakly (7 days after the start of co-culturing).

B1 and B2: CD68 detection. The morphologically monocytic cells with blue fluorescence indicated by arrows 1 were stained for CD68, but the morphologically vascular endothelial cell with blue fluorescence indicated by arrows 2 was barely stained. The cell with blue fluorescence that was beginning to morphologically change into an endothelial-like cell indicated by arrow 3 was weakly stained (3 days after the start of co-culturing).

C1 and C2: The morphologically vascular endothelial cells with blue fluorescence indicated by arrows 1 were stained for vWF. The cell with blue fluorescence that was morphologically flattened, indicated by arrow 2, was also stained. The morphologically monocytic cells with blue fluorescence indicated by arrows 3 were not stained (3 days after the start of co-culturing).

D1 and D2: VE-cadherin was stained between the morphologically vascular endothelial cell with blue fluorescence indicated by the arrow and the surrounding vascular endothelial cells (3 days after the start of co-culturing).

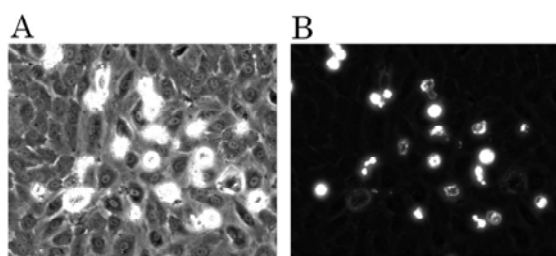


Figure 1.

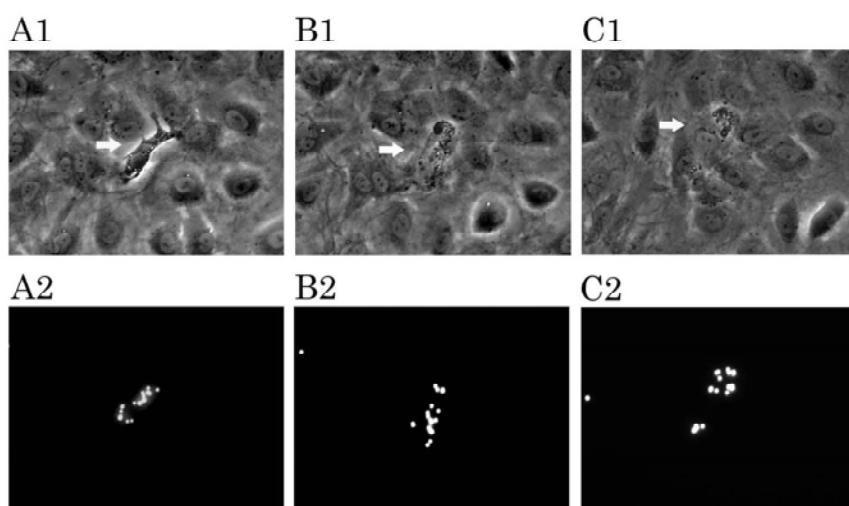


Figure 2.

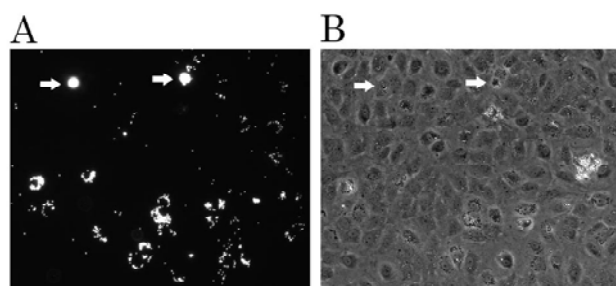


Figure 3.

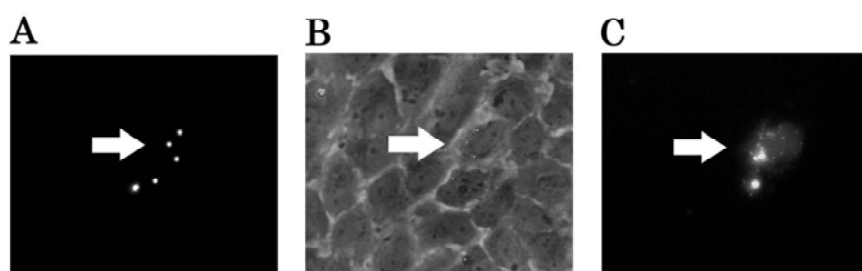


Figure 4.

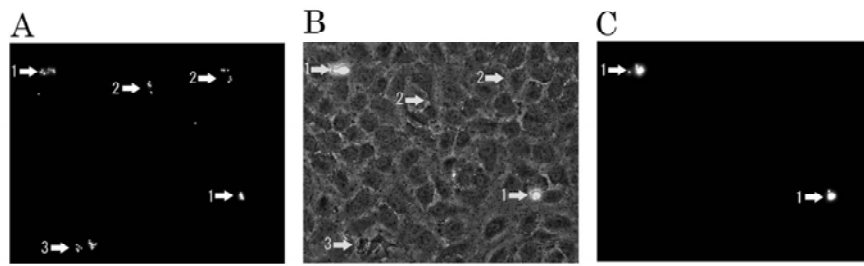


Figure 5.

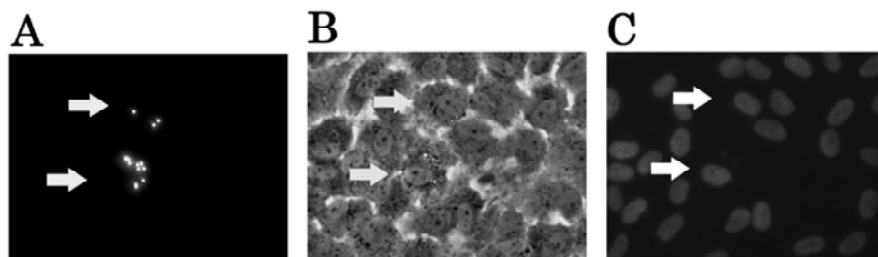


Figure 6.

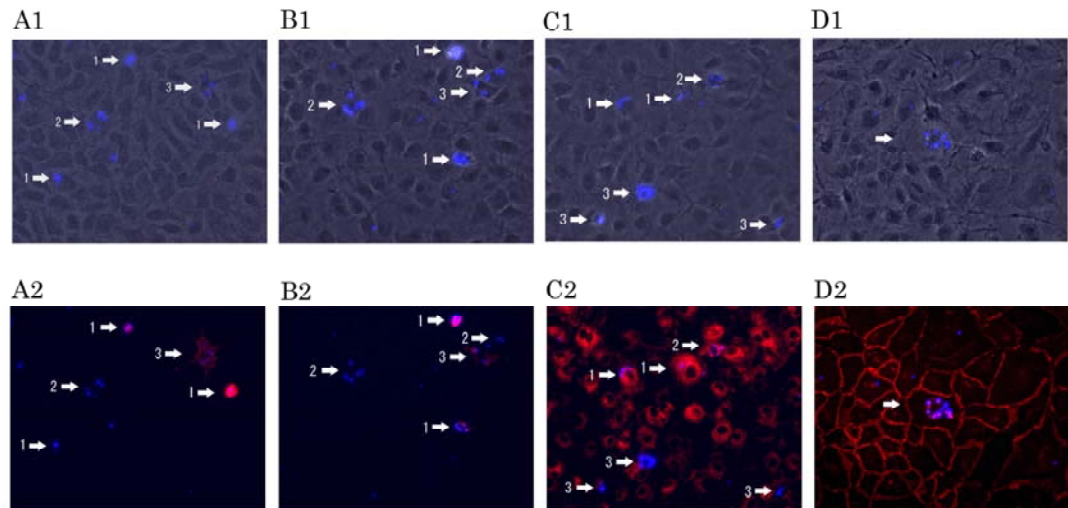


Figure 7.