이학석사 학위논문

Human Podoplanin-Positive Myeloid cells Acquire Lymphendothelial Characteristics and Enhance Lymphangiogenesis with Platelet through Podoplanin/CLEC-2 axis

포도플라닌 표지 혈구세포의 림프관내피세포 성질 획득과 혈소판과의 상호작용을 통한 림프관신생에 관한 연구

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Human Podoplanin-Positive Myeloid cells Acquire Lymphendothelial Characteristics and Enhance Lymphangiogenesis with Platelet through Podoplanin/CLEC-2 axis

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Abstract

Human Podoplanin-Positive Myeloid cells Acquire Lymphendothelial Characteristics and Enhance Lymphangiogenesis with Platelet through Podoplanin/CLEC-2 axis

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Background: Emerging studies suggested that murine podoplanin-positive myeloid cells are involved in lymphangiogenesis. However, in human conditions, we do not know whether podoplanin-positive myeloid cells could be obtained from peripheral blood, how they interact with platelet to achieve lymphangiogenesis, and how valuable they are therapeutically.

Methods and Results: Aggregation culture of human peripheral blood mononuclear cells (PBMCs) under high density using ultra-low attach dish resulted in cellular aggregates termed hematospheres. During 5-day hematosphere culture, podoplanin-positive myeloid cells expanded exponentially and expressed several lymphatic endothelial cell-specific markers including VEGF receptor-3 and lymphangiogenic transcription factors. Next, we investigated the potential interaction of podoplanin-positive cells with platelets that had C-type lectin-like receptor-2 (CLEC-2), a receptor of podoplanin. In vitro co-culture of podoplanin-positive cells and platelets stimulated monocytes to strongly express lymphatic endothelial markers and up-regulated lymphangiogenic cytokines. Recombinant human CLEC-2 also stimulated podoplanin-positive monocytes through Akt and Erk signaling. Likewise, platelets stimulated by co-cultured monocytes up-regulated lymphangiogenic cytokine IL-1beta. The supernatant of co-culture was able to enhance the migration, viability and proliferation of LEC. Local injection of podoplanin-positive monocytes with platelets significantly increased lymphatic neovascularization and facilitated wound healing in the full-thickness skin wounds of nude mice more than hematospheres alone did.

Conclusion: Co-treatment with podoplanin-positive monocytes and platelets augments lymphangiogenesis through podoplanin/CLEC-2 axis, which thus would be a promising novel strategy of cell therapy to treat human lymphatic vessel disease.

Keywords: Podoplanin, Peripheral blood mononuclear cell, Monocyte, Platelet, CLEC-2, Lymphangiogenesis

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Introduction

Disruption of the lymphatic system can cause chronic problems such as remodeling of the skin and the subcutaneous extracellular matrix, leading to the accumulation of lipids and macrophages in the affected tissue. Treatment of lymphatic vascular disease relies heavily upon an empirically-developed lymphatic-specific massage technique termed manual lymphatic drainage. However, care of this chronic condition require lifelong attention and good compliance as for other vascular diseases and diabetes. Also, it is well known that diabetic patients frequently have severe problems with impaired wound healing whose mechanisms are generally poorly understood. Therefore, new therapies to treat chronic lymphedema and impaired wound healing are in great demand.

Adult bone marrow or peripheral blood is most convenient therapeutic cell source and has been widely used in the treatment of various hematologic or ischemic diseases.

Previous report shows that murine monocyte/macrophages contribute to the formation of lymphatic vessels and promotes diabetic wound healing. Additional reports demonstrated that mouse bone marrow or circulating blood-derived cells that express lymphendothelial cell markers can function as lymphatic endothelial progenitor cells and participate in postnatal lymphatic neovascularization. Among these reports, we can infer that peripheral blood-derived lymphangiogenic cells, which are relatively easily-accessible, might be of therapeutic value in patients with chronic or acute lymphatic edema and impaired wound healing. However, the limitation of the previous studies is lack of evidence in human cells because the results were based on murine cell sources. Therefore, identification of human lymphangiogenic cells and evaluation of their lymphangiogenic potential are required. Also, breakthroughs to overcome the limiting factor of cell therapy for lymphatic diseases is needed since the number lymphangiogenic cells derived from peripheral blood or bone marrow is insufficient.

Recent studies suggested the association of monocytes and

lymphangiogenesis by demonstrating that aggregation of monocytes can lead to lymphatic vessel formation, and another study has implied that murine podoplanin-positive myeloid cells are crucially involved in lymphangiogenesis. Further investigations showed that the receptor of podoplanin, CLEC-2 is expressed on platelets that may regulate lymphatic vascular development. We applied this concept to our hematospheres that were generated by high cell density with suspended aggregation culture of human peripheral blood mononuclear cells (PBMCs). From these findings, we hypothesized that the human podoplanin-positive myeloid cells can participate in lymphatic neovascularization and that the interaction between podoplanin of these myeloid cells and CLEC-2 on platelets can potentiate the lymphatic neovascularization.

Materials and Methods

All study protocols in this study were approved by the Institutional Review Board (IRB) and Institutional Animal Care and Use Committees (IACUC) of Seoul National University Hospital.

Preparation of human hematospheres and platelets

We prepared human peripheral blood mononuclear cells (PBMCs) from healthy donors with informed consent as described previously with minor modifications [1]. Isolation of PBMCs was done by Ficoll-Paque^{TM} PLUS (GE healthcare) according to instructions and PBMCs were washed five times with phosphate buffered saline (PBS) to completely remove remaining debris. PBMCs suspended in StemSpan® H3000 (StemCell Technologies) or mTeSR (StemCell Technologies) were cultured at 3-5x10^6 cells/ml on Hydrocell^{TM} Ultra-Low attach surface (NUNC). One ml of fresh medium was added every second day without media change. Isolated mononuclear

cells started to aggregate after 1 day of culture, and these aggregates were termed as hematospheres. The number of hematospheres increased during 5 days of culture, with non-incorporated single cells dispersed in the periphery.

Platelets were isolated as previously described [2]. Human blood was drawn into acid-citrate-dextrose (ACD) coated vacuum tube and centrifuged (200*g* for 20 min) to obtain platelet-rich plasma (PRP). PRP was centrifuged (500*g* for 20 min) in the presence of 100 nM PGE-1 (Sigma) and washed with pipes/saline/glucose containing 100 nM of PGE-1. The isolated platelets were either quiescent or allowed to adhere to immobilized human fibrinogen (Sigma) in the presence of thrombin (Sigma) or rh-podoplanin (ProSpec Bio).

hLECs (PromoCell) were maintained in endothelial cell basal medium-2 (EBM-2) supplemented with cytokine cocktail (SingleQuots; Lonza).

Acquisition of Conditioned Media and Measurement of Cytokine Concentration

After 2days of cell culture, conditioned medium was acquired from each group by removing cellular debris via centrifuging and were stored at −70° C until the analysis. The concentration of VEGF−A, HGF and IFN gamma were analyzed by Bio−Plex® 200 System (Bio−Rad Laboratories) according to the manufacturer's protocol. Anti−VEGF−A, HGF and IFN gamma antibodies were purchased from Bio−Rad.

Scratch Wound Assay

Scratch wound assay was performed as described previously [3]. hLECs were grown in 35 mm dishes to a confluent monolayer. The hLEC monolayer was scraped in a straight line with a 200 μL pipet tip. After wounding, monolayers were immediately washed and incubated with each conditioned medium. After 20 hours, migrated cell number from wound edge to point of cell migration was measured.

Viability Assay

Cell viability was carried out as previously described [3]. For assessment of viability, $1x10^4$ hLECs were seeded to each

well of 96-well plate in EBM-2 with 1% FBS. Then, the cultured media were changed with each conditioned medium. After 48 hours of incubation, reagent WST-1(Roche Molecular Biochemicals) was added with cell culture medium as 20μL/well. The cells were incubated for 2 to 4 hours in the same incubator. Absorbance was measured using a microplate reader (SpectraMax 190) at 450 nm.

Proliferation Assay

hLECs were seeded on 96-well plates at a concentration of 1×10^4 cells/well in EBM-2 with 5% FBS and stabilized for 12 hours. After overnight culture in EBM-2 with 1% FBS, hLECs were incubated in each conditioned medium. Then, the cells were treated with BrdU for 24 hours. Incorporated BrdU was detected with anti-BrdU monoclonal antibodies conjugated with peroxidase. Sample absorbance was analyzed using a microplate reader (SpectraMax 190) at 450nm.

Matrigel Tube Formation

In vitro tube formation was evaluated using the Matrigel plate

as described previously [4]. Matrigel (Becton Dickinson Labware) basement membrane matrix was added to 4-well chamber slide. After 30min of incubation at 37℃, each cells were seeded in EBM-2 with 5% FBS. 12 hours later, four representative fields were taken.

Skin and Ear wound model

Wounds were created in nude mice 8 to 10 weeks of age as described previously [5]. Full-thickness and excisional skin wounds were created on the backs of the mice using 6-mm skin biopsy punches. Ear wounds were created by using ear punch with 2-mm diameter. Each cells were administered intradermal around the wounds, and wounds were covered with a sterile transparent occlusive dressing (Tegaderm; 3M Health Care). Wound areas were measured and digital photographs were taken 0, 3, 5, 7 days. Seven days later, the wounds were carefully excised and fixed in 4 % paraformaldehyde and embedded in paraffin or OCT compound.

Image Acquisition and Analysis

Images were obtained by an Olympus IX2 inverted fluorescence microscope (Olympus) equipped with an Olympus DP50 CF CCD camera and analySIS 5.0 software. Confocal images were obtained by Zeiss LSM-710 META confocal microscope (Olympus) and ZEN 2009 analysis software.

Statistical Analysis

All data were presented as means ± S.E.M. The statistical significance of difference between two groups was evaluated with an unpaired t-test and the significance between three groups was analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's method using the Prism 4 program (GraphPad). Probability values less than 0.05 were considered significant.

Results

Aggregation Culture of Human Peripheral Blood Mononuclear Cells Induces Expansion of Podoplanin-positive Cells and Up-regulation of Lymphangiogenic Genes

To investigate whether human monocyte aggregates have lymphangiogenic potential, we first examined LEC marker expression in the hematospheres during 3D culture by fluorescence associated cell sorter (FACS) analysis and western blot. First, freshly isolated PBMCs were cultured on the ultra-low attach dish as previously described [1]. As a result, numerous cellular spheroids, which we call as hematospheres, were observed via 3D culture (Figure 1A). During 3 days of culture, the expression of podoplanin increased dramatically in hematospheres compared to freshly isolated PBMCs, and about 75% of monocytes expressed podoplanin at day 5 (Figure 1B and 1C). Next, we sought to determine the optimal culture media for expansion of podoplanin-positive cell in hematospheres and to confirm the specificity of podoplanin on these cells by screening several

different antibodies. As a result, we selected the StemSpan and mTeSR as 3D culture media because of superior amplification of podoplanin-positive cells than EBM-2 with 5% FBS. Additionally, we confirmed that hematospheres have podoplanin-positive cells by using different clones of podoplanin antibodies in FACS. Despite clonal differences of antibodies, similar percentages of cells were positive for podoplanin in both antibodies. After optimization of experimental conditions, we compared the protein expression of podoplanin and VEGFR-3, another maker of lymphatic endothelial cell, between freshly-isolated PBMCs and hematospheres as well as in hLEC. Podoplanin and VEGFR-3 were significantly increased by 3D culture (Figure 1D). In addition, we found that most cells constituting hematosphere were stained with podoplanin and VEGFR-3 by the immunofluorescence (Figure 1E). To explore whether hematospheres express lymphangiogenesis-related genes, we conducted semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Hematospheres showed a significant induction of lymphatic endothelial cell-

specific genes, such as membrane bound molecules (Podoplanin, VEGFR-3, and Ephrin-B2), transcription factors (Prox-1, Sox-18, and FoxC-2), and cytokines or growth factors (Angiopoietin-2, VEGF-A, and VEGF-D) (Figure 1F). On the other hand, expression of TGF-β1, anti-lymphangiogenic cytokine, decreased gradually and VEGF-C maintained low level until day 5 [6-8]. Interestingly, the expression of Angiopoietin-1 decreased during culture while expression of Angiopoietin-2 increased. These results indicated that hematosphere provided a good niche for myeloid cells to trans-differentiate and to get the characteristics of lymphatic endothelial cell.

Podoplanin-positive/VEGFR-3^{high} Myeloid Cells in Hematospheres Possess Lymphangiogenic Characteristics whereas Podoplanin-negative/VEGFR-3^{low} Myeloid Cells does not.

To characterize the podoplanin-positive cells in hematospheres, we performed fluorescence associated cell sorting. Specifically, we isolated the podoplanin-positive or

the podoplanin-negative cells from the 5-day-cultured hematospheres which were mainly composed of $CD14^+$ myeloid cells. Podoplanin-positive myeloid cells showed strong expression of VEGFR-3 whereas podoplanin-negative myeloid ones showed low levels (Figure 2A). Quantitative RT-PCR of sorted cells demonstrated that the podoplanin-positive cells had significantly higher expression of Podoplanin (40-fold), VEGF-C (3-fold), Prox-1 (4-fold), Sox-18 (2-fold), and FoxC-2 (6-fold) the podoplanin-negative cells did (Figure 2B), whereas expression of VEGFR-3 (1.2-fold), Ephrin-b2 (1.3-fold), and VEGF-A (1.2-fold) showed insignificant difference.

Furthermore, to examine the distinct characteristic of hematospheres and non-hematosphere single cell fractions (not incorporated into hematospheres), we separated them into two populations and cultured them in EBM-2 with 5% FBS on 1.5% gelatin coated dish. While dissociated single cells from hematosphere fraction attached to the dish surface and displayed spindle-shaped morphology, those from non-hematosphere fraction remained suspended in culture. These

attached cells from hematosphere fraction were positive for both podoplanin and VEGFR-3 in immunofluorescence analysis (Figure 2C), and were able to participate in the growth of lymphendothelial tube-like structure with lenti-GFP transduced hLECs on thin Matrigel tube formation assay (Figure 2D).

The Interaction between Podoplanin-Positive Monocytes and Platelets Potentiates Lymphangiogenic Characteristics of Podoplanin-Positive Monocytes

To examine the interaction between podoplanin-positive monocytes and platelets, we co-cultured podoplanin-positive monocytes and platelets. Podoplanin-positive monocytes were dissociated to single cell state to enhance contact with platelets (Figure 3A). To identify the role of podoplanin in the interaction between podoplanin-positive monocytes and platelets, we used blocking antibody against podoplanin. Podoplanin-positive monocytes were pretreated to podoplanin blocking antibody or their isotype antibody before co-culturing with platelets.

When exposed to platelets, podoplanin-positive monocytes were remarkably committed to lymphatic cells with significant induction of LEC-specific markers, such as Podoplanin, Prox-1, Sox-18, FoxC-2, VEGF-A, VEGF-C, and Angiopoietin-2 in quantitative RT-PCR. Their up-regulated expressions were reversed when they were pretreated with podoplanin blocking antibody (Figure 3B). In the ELISA assay, the representative lymphangiogenic cytokines such as VEGF-A [9] and HGF [10] significantly increased in the supernatant of podoplanin-positive monocytes co-cultured with platelet, which was attenuated in the presence of the podoplanin blocking antibody (Figure 3C). Intriguingly, INF-gamma [11] as anti-lymphangiogenic cytokine decreased in the supernatant of podoplanin-positive monocytes exposed to platelets, which was also reversed by podoplanin blocking (Figure 3C).

To investigate the signalling mechanism of lymaphatic committment of myeloid cells by exposure to platelets, which was depedent on podoplanin, we stimulated podoplanin-positive myeloid cells from hematosphere with recombinant

human CLEC-2 (rhCLEC-2), counter partner of podoplanin [12]. We checked the PI3K/Akt and Erk pathway which have been reported to involved in the transdifferentiation of LECs [13] or maturation of lymphatic valve and vessels in development [14]. Treating podoplanin-positive monocyte with rhCLEC-2 increased the activation of Akt and Erk while treatment of podoplanin blocking antibody attenuated this effect (Figure 3D). Inhibition of PI3K by LY294002 blocked the phosphorylation of Akt and the inhibition of MEK by PD98059 attenuated the phosphorylation of Erk significantly, whose results were similar to the podoplanin blocking antibody treatment. These results indicated that interaction between podoplanin-positive monocytes and platelets facilitated lymphatic commitment of myeloid cells through podoplanin / CLEC-2 axis followed by activation of PI3K/Akt and Erk pathways.

Podoplanin Activates Platelets, Leading to Induction of IL-1beta.

Next the change in platelets was evaluated during interaction

with podoplanin-positive monocytes. The most dramatic change in platelets was enlargement of platelets in co-culture condition, compared with platelets in single culture condition. This enlargement of platelets was remarkably prevented by podoplanin-blocking antibody (Figure 4A and 4B).

Then, to dissect the underlying mechanism for the activation of platelets by podoplanin-positive monocytes, we directly applied recombinant human-podoplanin (rh-podoplanin) on platelets. We cultured the platelets that were allowed to adhere to immobilized human fibrinogen in the presence of rh-podoplanin or thrombin as a control stimulant. Cellular spreading on immobilized fibrinogen in the presence of rh-podoplanin demonstrated that platelets were efficiently stimulated, and the average area of platelets significantly more increased in presence of rh-podoplanin in the similar way to thrombin, whereas control group had no effect. Second, we examined the level of the IL-1beta in the activated platelets, which was known as a potent lymphangiogenic cytokine [15]. Immunofluorescence staining showed IL-1beta protein was up-regulated in the activated platelets by rh-

podoplanin or thrombin while CLEC-2 protein constitutively expressed in the platelets (Figure 4C). The increased gene expression of IL-1beta in the activated platelets by rh-podoplanin was also confirmed by semi-quantitative RT-PCR analysis (Figure 4D). In other words, the rh-podoplanin could activate platelets and increase IL-1beta at mRNA and protein level in platelets even without nucleus as strongly as thrombin. Taken together, the co-culture of podoplanin-positive monocytes and platelets may not only induce lymphatic commitment of monocytes but also activate platelets. These reciprocal effects were mediated through the interaction between podoplanin and CLEC-2 axis.

Co-culture Supernatant of Podoplanin-Positive Monocytes with Platelets Augments the Migration, Viability, and Proliferation of Lymphatic Endothelial Cells

To investigate the in vitro paracrine effect of podoplanin-positive monocytes with platelet on hLEC, we performed functional assays of hLEC using the co-culture supernatant. We found that supernatant of podoplanin-positive monocytes

with platelet increased migration, viability, and proliferation of hLEC compared with supernatant of either platelet or podoplanin-positive monocyte (Figure 5A-C). All these increases by co-culture supernatant were attenuated by podoplanin blocking antibody in the co-culture. These data provided ex vivo evidence that podoplanin-positive monocytes when exposed to platelet exert stimulatory paracrine action on hLEC.

Human Podoplanin-Positive Monocytes with Platelets Augment Lymphatic Vessel Formation and Wound Healing in the Nude Mice

Based on the data that podoplanin-positive monocytes exposed to platelet are committed to lymphatic endothelial cells and augment the lymphangiogenic capability of hLEC, we investigated the effect of human hematosphere-derived podoplanin-positive monocytes with platelet on the enhancement of lymphangiogenesis in the nude mouse dorsal skin and ear wound model. We compared the wound-healing efficacy of 5 different treatments by serial measurements of

dorsal skin wound at days 0, 3, 5 in nude mice: (1) PBS, (2) platelet, (3) podoplanin-positive cell, (4) podoplanin-positive cell with platelet, (5) podoplanin-blocking antibody-treated podoplanin-positive cell with platelet. Local injection of podoplanin-positive monocytes with platelet remarkably facilitated wound healing as short period as 5 days, which was significantly different from the other treatments; PBS, platelets or podoplanin-positive monocytes. Such a facilitation of wound healing by podoplanin-positive monocytes with platelet was attenuated by podoplanin-blocking antibody (Figure 6A and 6B).

The mechanism of the facilitated wound healing by podoplanin-positive monocytes with platelet was revealed by histologic examination as increase of lymphatic vessel formation around dorsal skin wound. The number of LYVE-1 positive vessel was greater by treatment of podoplanin-positive monocytes with platelet more than by podoplanin-positive monocytes only, which was again attenuated by podoplanin-blocking antibody (Figure 6C and 6D). In addition to number, lymphatic vessel sprouting was also more

prominent by the treatment of podoplanin-positive monocytes with platelet in the whole-mount immunostaining of ear wound. The number of lymphatic vessel branching points was significantly increased by podoplanin-positive monocytes with platelet than podoplanin-positive monocytes alone, which was diminished by podoplanin-blocking antibody (Figure 6E and 6F).

To further investigate the localization of injected human podoplanin-positive monocytes, we established the immunostaining of human specific antibody, Human Leukocyte Antigen-ABC (HLA-ABC), and found that podoplanin-positive monocytes were either located adjacent to mouse lymphatic vessel or directly incorporated into lymphatic vasculature (Figure 6G). Together, these data demonstrated that human podoplanin-positive monocytes derived from hematosphere, with the aid of platelet, were effective in wound repair by stimulating lymphatic vessel formation and sprouting, which was dependent on podoplanin-CLEC2 interaction.

Discussion

In this study, we have shown that lymphatic endotheloid cells positive for both podoplanin and VEGFR-3 could be remarkably expanded by the short period of three dimensional (3D) aggregation culture (Hematosphere) of human peripheral blood myeloid cells. Previous discoveries of podoplanin-positive cells were limited to murine cells obtained from bone marrow or peripheral blood. Our research reveals that co-culture of human podoplanin-positive monocytes with platelets triggers the lymphangiogenic signaling by reciprocally-stimulating each other, specifically, by the interaction of podoplanin on the surface of the human myeloid cells and CLEC-2 molecule on platelets through Akt and Erk signaling. Podoplanin-positive monocytes with platelet showed the marked increase of the secretion of various lymphangiogenic cytokines, leading to activation of migration and proliferation of hLEC, and then to the enhanced lymphangiogenesis around skin wound with facilitated healing.

These data show that human podoplanin-positive monocytes are easily obtainable from hematosphere and are promising new cell sources when combined with platelets for the treatment of chronic lymphatic disorders or diabetic wound healing.

Aggregation-culture of human monocytes generates lymphatic endotheloid cells within the hematosphere

Previous study by Maruyama K. et al suggested that lymphangiogenesis can occur by mouse monocyte aggregates that transdifferentiate into lymphatic endothelial cells [16]. With this study in mind, we investigated whether we could get the lymphatic endothelial cells from human monocytes' aggregates expanded by our hematosphere culture method. Upon closer examination of these human peripheral blood monocytes aggregates, we discovered interesting evidence that human myeloid cells constituting these aggregates gradually increased the expression of lymphatic endothelial cell markers such as podoplanin and VEGFR-3 during hematosphere culture. Gene expression analysis showed that

hematospheres at day 5 expressed mRNAs of Prox-1 and Sox-18. Prox-1 is a master control gene in the program specifying lymphatic endothelial cell fate [17] and sox-18 is known to induce development of the lymphatic vasculature in mice [18]. Furthermore, we observed a sustained increase in Angiopoietin-2 expression and a decrease in Angiopoietin-1 in hematospheres at day 5, suggesting that podoplanin-positive monocytes exert lymphangiogenic effect via Angiopoietin-2 instead of employing Angiopoietin-1-mediating pathway [19, 20]. Additional in vitro Matrigel tube forming assay confirmed that these cells positive for both podoplanin and VEGFR-3 exhibited lymphangiogenic capability.

In essence, our aggregation culture method is similar to the de novo lymphangiogenesis of the mouse cornea, especially when previous report of mouse bone marrow derived macrophages transdifferentiating into LECs through aggregation is taken into account [16]. We postulate that in a similar manner, human monocytes obtain lymphangiogenic capabilities by activation through in vitro aggregation culture. These results

indicate that for the first time, it is possible to obtain lymphatic endothelial cells from human peripheral blood using aggregation culture.

Podoplanin and VEGFR-3 positive myeloid cells differentiate into lymphangiogenic population

Podoplanin and VEGFR-3 are the distinct markers that are not shared with the endothelial cells of blood vessels [6]. Also, previous studies showed that factors such as VEGFR3 and CD133 in human stem-progenitor cells [21] or bone-marrow derived podoplanin positive cells in mice are critical for lymphangiogenesis [22]. Our analysis by aggregation culture suggested that in human peripheral derived monocytes, VEGFR-3^{high}/Podoplanin-positive population showed stronger lymphangiogenic potential than VEGFR-3^{low}/Podoplanin-negative population.

As shown in Figure 2B and 2C, podoplanin-positive cells of hematospheres expressed the distinct lymphatic marker, VEGFR-3, whereas podoplanin-negative cells of hematospheres expressed it at a very low level. The mRNA

expression of lymphangiogenic genes such as VEGF-C, Prox-1, Sox-18, and FoxC-2 was higher in VEGFR-3^{high}/Podoplanin-positive cells than that in VEGFR-3^{low}/Podoplanin-negative cells. On the basis of these results, we suggest that identifying characteristics of lymphangiogenic cells in human circulation system should be assigned as podoplanin and VEGFR-3 double positive expressing cells, rather than VEGFR-3 positive cells.

Reciprocal stimulation between podoplanin of monocytes and CLEC-2 molecules of platelets

During embryonic development, lymph sacs form from the cardinal vein, and sprout centrifugally to form mature lymphatic networks. Unrin et al. suggested that the interaction of endothelial podoplanin of the developing lymph sac with blood platelets might be important for lymphangiogenesis [23]. With previous studies describing the interaction of cell surface marker podoplanin with platelet-associated molecule C-type lectin-like receptor-2 (CLEC-2), we decided to further investigate whether expanded myeloid cell's lymphatic

potential could be augmented by the reciprocal interaction with platelets. To study the role of interaction platelets and podoplanin-positive monocytes, we analyzed reciprocal effects between platelets and podoplanin-positive monocytes, with the following observations;

First, platelets augmented the expression of lymphangiogenic genes in podoplanin-positive monocytes, suggesting that platelets might increase the lymphangiogenic potency of podoplanin-positive monocytes. In this process, CLEC-2 on platelet interacts with podoplanin and turn on the Akt and Erk pathways in monocytes.

Second, podoplanin-positive monocytes activated platelets, thereby leading a significant morphologic change of platelets and induction of IL-1beta which was known as a potent lymphangiogenic cytokines [15]. This was reproduced by the recombinant podoplanin that activated platelets also. With previous studies suggesting that platelets have mechanisms for up-regulating IL-1beta [24] and other genes [25] by pre-mRNA splicing when external stimuli are given [2], we proved that recombinant human podoplanin and podoplanin

positive monocytes up-regulate IL-1beta by stimulating CLEC-2 on platelets.

Third, podoplanin-blocking antibody blunted the reciprocal effects mentioned above between platelets and podoplanin-positive monocytes. On the basis of these data, we assumed that the podoplanin might be a key molecule in interaction between platelets and podoplanin-positive monocytes and that the reciprocal effects between platelets and podoplanin-positive monocytes might play an important role in lymphangiogenesis.

Therapeutic lymphangiogenesis by synergistic interaction between human podoplanin-positive monocytes and platelets: A promising strategy in treating lymphatic vessel diseases

To examine the role of podoplanin-positive monocytes with platelet on the lymphangiogenesis, we performed in vitro and in vivo experiments. The supernatant of podoplanin-positive monocytes with platelets significantly enhanced the migration and proliferation of hLECs, compared with supernatant of either cell alone. This enhanced effect was obliterated by

podoplanin-blocking antibody. On the basis of these data, we assumed that podoplanin-positive monocytes with platelets augment lymphangiogenesis via podoplanin/CLEC-2 axis. We investigated whether combination of human platelets with podoplanin-positive monocytes augments lymphatic neovascularization in nude mice skin wound model. We confirmed that the platelets play an important role to enhance lymphangiogenesis and facilitate wound repair, because treatment effect of only podoplanin-positive monocytes was less than the combined cell therapy. In the histologic examination, the therapeutic effects were displayed in several ways; (1) increased number of lymphatic vessels, (2) enhanced maturation or arborization of lymphatic vessels, and (3) direct incorporation to lymphatic vessels. This suggested that interaction between platelets and podoplanin-positive monocytes is necessary and that podoplanin-positive monocytes may trigger lymphangiogenesis through two ways; by direct incorporation into lymphatic vessels or by paracrine factors released from the reciprocal interaction between monocytes and platelets. Intriguingly, the interaction of

monocytes and platelets has been described as a physiologically-occurring phenomenon termed as monocyte-platelet aggregation (MPA) [26]. Although recent studies suggested MPA as a marker for various cardiovascular disease mechanisms, the mechanisms underlying this phenomenon remain mostly unknown. With our new evidence of monocyte-platelet interaction contributing to lymphangiogenesis, further studies will be needed to investigate the possible relationship between MPA and lymphatic neovascularization.

Conclusion

Our results indicate that podoplanin-positive monocytes have a potential of lymphangiogenesis, which can be augmented by platelets through interaction of podoplanin on monocytes and CLEC-2 on platelets. We found an easy and practical way to expand human podoplanin-positive monocytes through hematosphere culture that is a high-density 3D suspension culture of human peripheral blood monocytes. Therefore, combined cell therapy using podoplanin-positive monocytes and platelets might become a promising way to induce lymphatic neovascularization and facilitate wound healing.

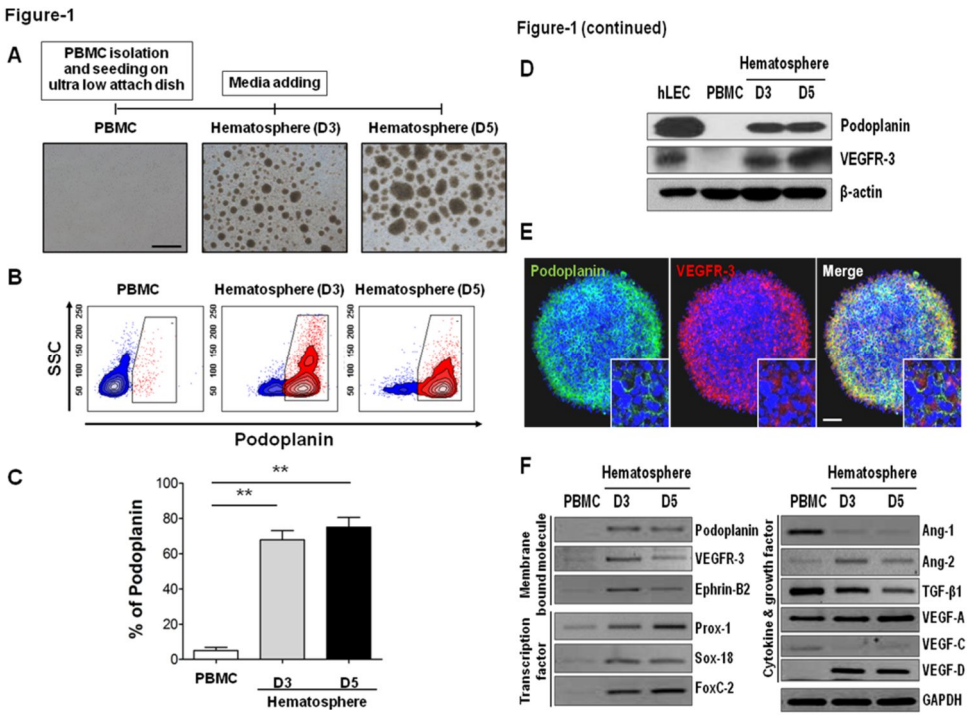


Figure 1. Aggregation culture allows myeloid cells to obtain the characteristics of lymphatic endothelial cell (LEC).

(A) Timeline of aggregation culture. Peripheral blood mononuclear cells (PBMCs) were cultured under three-dimensional condition and harvested at day 3 or 5. Scale bar= 0.5mm. (B) Flow cytometry analysis of fresh PBMCs and cultured hematospheres at day 3 and 5 were performed with podoplanin antibody. (C) Bar graph representing the percentage of podoplanin-positive cells during hematospheres culture. Each value is the average of 3 independent experiments (**P<0.01; n=4 per experiment). (D)

Protein expression analysis of podoplanin and VEGFR-3 in hLEC, fresh PBMCs, and cultured hematospheres at day 3 and 5 via western blot analysis. Beta-actin was utilized as an internal control. (E) Whole mount immunofluorescence staining of the cultured hematospheres on day 5 with podoplanin and VEGFR-3. Scale bar= 50 μm. (F) Gene expression analysis of lymphangiogenesis-related genes in fresh PBMCs and cultured hematospheres at day 3 and 5 via semi-quantitative PCR. GAPDH was utilized as an internal control.

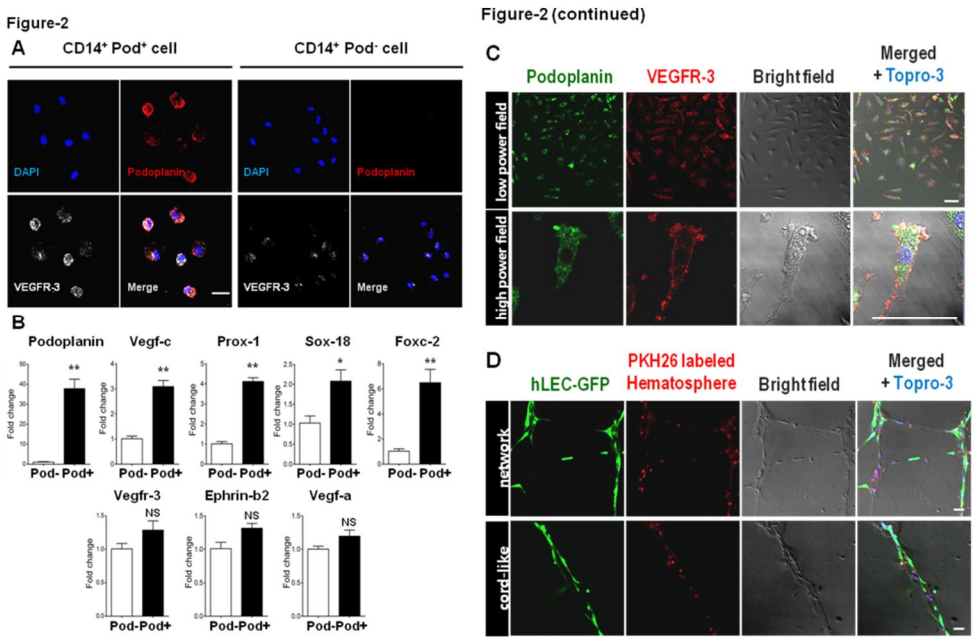


Figure 2. Podoplanin-positive/VEGFR-3^{high} myeloid cells within hematospheres express lymphangiogenic characteristics.

(A) Flow cytometry-sorted CD14$^+$ Pod$^+$ cells and CD14$^+$ Pod$^-$ cells from day 5 hematospheres were subjected to immunofluorescence with podoplanin and VEGFR-3. Scale bar= 20 μm. (B) Quantitative RT-PCR of flow cytometry-sorted CD14$^+$ Pod$^+$ cells and CD14$^+$ Pod$^-$ cells. Bar graphs represented the relative quantity of the lymphangiogenesis-related genes expression in the pod$^+$ cells compared to pod$^-$ cells. (**P<0.01, *P<0.05; n=3 per experiment) (C) Dissociated single cells from hematospheres were seeded on 1.5% gelatin coated dish and

cultured for 24hours. Attached cells were subjected to immunofluorescence image with podoplanin and VEGFR-3. Scale bar= 50㎛. (D) Co-culture PKH26 labeled dissociated single cells from hematospheres with lenti-GFP transduced hLEC on matrigel. Scale bar= 50㎛.

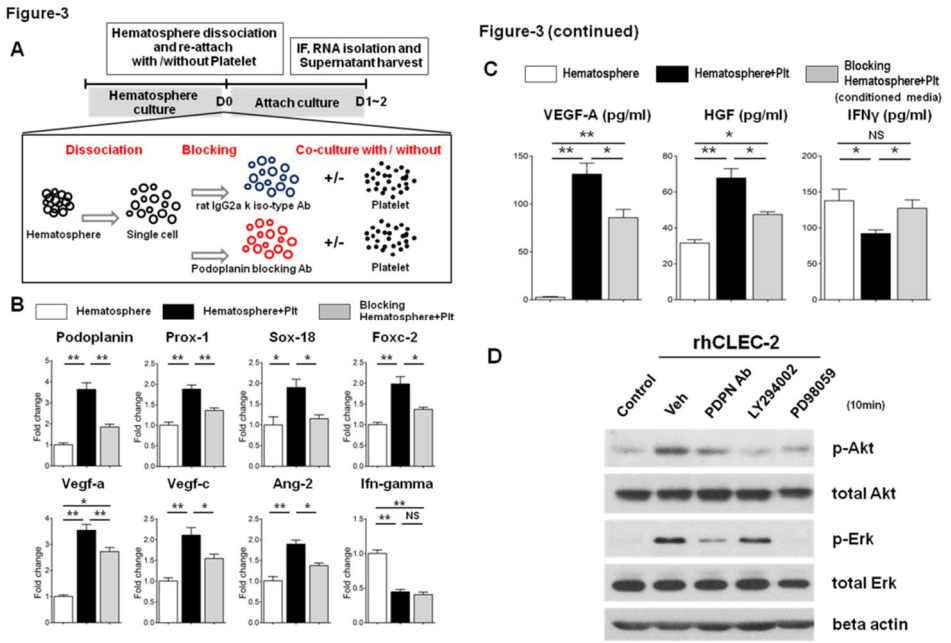


Figure 3. Human podoplanin-positive monocytes and platelets reciprocally activate each other via podoplanin/CLEC-2 axis.

(A) Experimental scheme for coculture of podoplanin-positive monocytes and platelets. Cultured podoplanin-positive monocytes were dissociated to single cells and pretreated with podoplanin blocking or isotype antibody (100 μg/ml) for 30 minutes. Cells were then divided into two groups, one receiving freshly isolated platelets while the other was treated with vehicle. Both groups were culture for 24-48 hours and both culture supernatant and RNA were harvested. (B) Quantitative RT-PCR of podoplanin-positive monocytes under single culture versus co-cultured with platelets in

the presence of isotype or podoplanin-blocking antibody. Bar graphs represent the relative quantity in the lymphangiogenesis-related genes. Coculture with platelets significantly induced the expression of lymphangiogenic gene expression in monocytes, which was remarkably prevented by blocking podoplanin-CLEC2 interaction. (**P<0.01, *P<0.05; n=3 per experiment) (C) Enzyme-linked immunosorbent assay (ELISA) for lymphangiogenesis-related cytokines (VEGF-A and HGF) and anti-lymphangiogenesis-related cytokine (IFN gamma) in each conditioned medium of monocytes. (D) Analysis of cell signaling in podoplanin-positive monocytes after stimulation with CLEC-2. Stimulation of monocytes with rhCLEC-2 led to activation of Akt and Erk in Western blot analysis, which was partly prevented by podoplanin-blocking antibody; starvation media (Control), DMSO (Veh), podoplanin blocking antibody (PDPN Ab), PI3K inhibitor LY294002 (LY294002) or MEK inhibitor PD98059 (PD98059) treatment for one hour prior to stimulation with rhCLEC-2 (5㎍/ml, 5min).

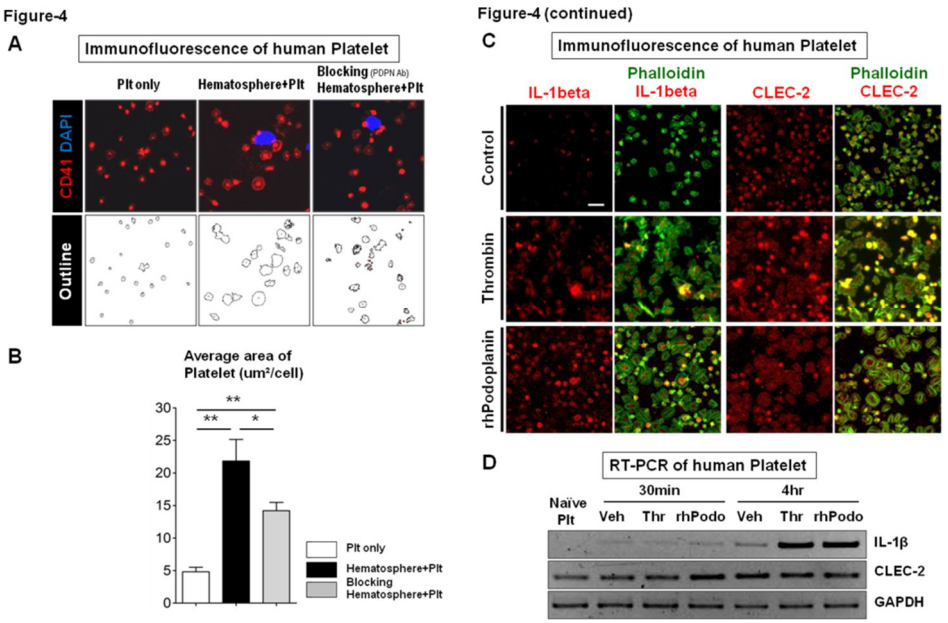


Figure 4. Human podoplanin-positive monocytes or Rh-podoplanin activates platelets and increases the accumulation of IL-1beta in platelets.

(A), (B) Platelets were left in a quiescent state or were co-cultured with podoplanin-positive monocytes in the presence of isotype or podoplanin-blocking antibody (100 μg/ml) on immobilized fibrinogen (200 μg/ml). After 2 hours, cells were fixed, and stained with nuclei for monocytes (blue) and CD41 for platelets (red). Coculture with podoplanin-positive monocytes significantly enlarged the size of platelets, which was remarkably prevented by podoplanin-blocking antibody. (B) Bar graph representing the total

area of platelets (**P<0.01, *P<0.05; n=3). (C) Platelets were activated by rh-podoplanin (20㎍/ml), with thrombin (0.01U/ml) and vehicle being used as positive and negative control. Platelets in each group were stained for IL-1beta or CLEC-2 (red). The green fluorescence represents polymerized actin, Phalloidin. Podoplanin significantly activated platelets resulting in the induction of IL-1 beta expression and morphologic change, as thrombin did. Scale bar= 10㎛. (D) After platelets were allowed to adhere to immobilized fibrinogen for 30min and 4hours, expression of IL-1beta and CLEC-2 in each group was determined by semi-quantitative PCR. Rh-Podoplanin significantly induced the lymphangiogenic cytokine IL-1 beta in platelets as thrombin did.

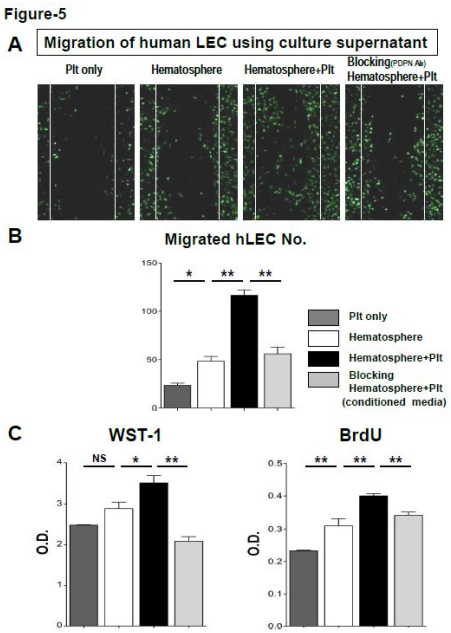
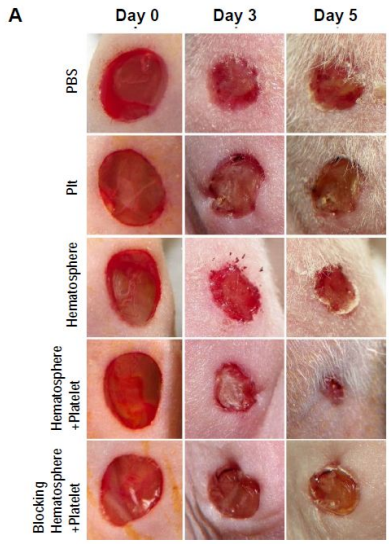


Figure 5. Coculture supernatant from podoplanin-positive monocytes and platelets enhances the migration, viability, and proliferation of human lymphatic endothelial cells. (A) Representative figure of hLEC migration in various conditioned media. hLEC migrated most rapidly in the presence of the conditioned medium from coculture of podoplanin-positive monocytes and platelet, while migration significantly decreased when treated with blocking antibody against podoplanin. (B) Bar graph representing the migrated cell number of hLEC (**P<0.01, *P<0.05; n=3 per experiment). (C) Quantitative data of viability and proliferation formed by hLEC under various conditions. hLEC

cultured under the conditioned medium from coculture of podoplanin-positive monocytes and platelets showed increased viability and proliferation compared to those in the culture mediums from monoculture of platelets or podoplanin-positive monocytes. However, the enhanced capacity of hLEC was attenuated by blocking the podoplanin. (**P<0.01, *P<0.05; n=3 per experiment)

Figure-6



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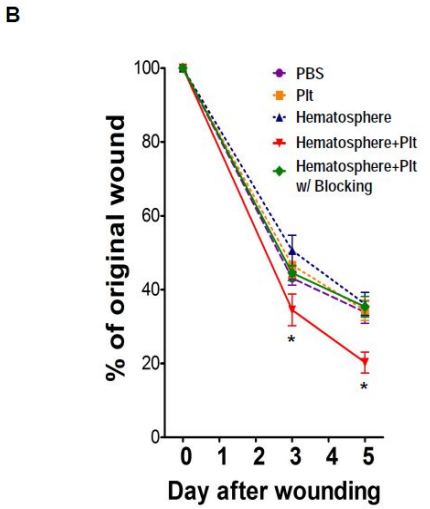

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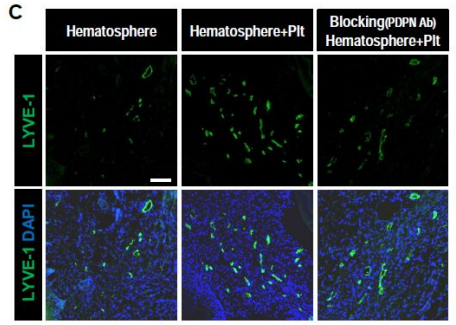

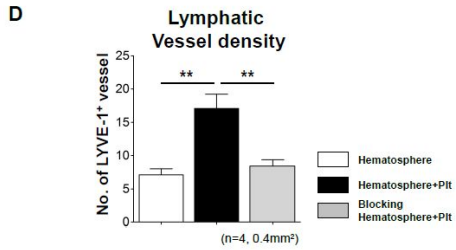

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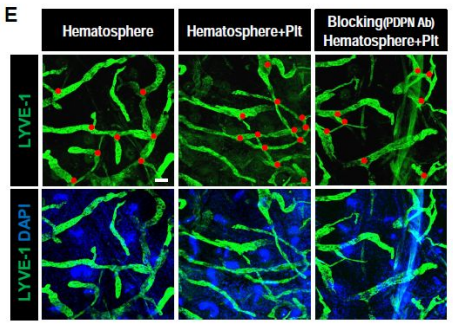

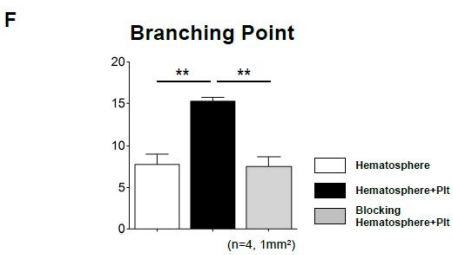

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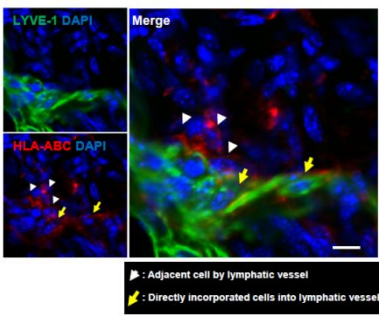

Figure 6. Synergistic interaction of human podoplanin-positive monocytes and platelets led to lymphatic neovascularization and facilitated wound healing in nude mice. (A) Gross appearances of the wounds at the back of nude mice injected with the different cells at the indicated time points. (B) Diagram of the kinetics of wound closure in each group. Four mice were analyzed at each time point (*P<0.05). (C to G) Mice that had received surgery for wound to back or ear skin were injected with the different cells, and the tissues were harvested at 7 days for immunohistochemistry. (C) In the confocal images of back skin from nude mice of indicated group, the LYVE-1 positive lymphatic vessels were more abundant after transplantation of platelet and podoplanin-positive monocytes than monocytes alone, which was reversed by blocking antibody against podoplanin. Scale bar= 100 μm. Blue fluorescence indicates DAPI. (D) Bar graph representing the quantification of lymphatic vessel number in the back skin which was determined by scoring LYVE-1 positive vessels. (**P<0.01; n=4 per experiment) (E) Whole-mount immunostaining of ear skin stained with antibodies against LYVE-1. Scale bar= 100 μm. Blue fluorescence indicates DAPI. Red dots denote branching points. LYVE-1 positive lymphatic vessels arborized more abundantly after transplantation of platelet

and podoplanin-positive monocytes than monocytes alone, which was reversed by blocking antibody against podoplanin. (F) Bar graph represented the number of lymphatic vessels branching points. (**P<0.01; n=4 per experiment) (G) In confocal images of skin wound treated with podoplanin-positive cells, about half of these transplanted human cells as stained red (HLA-ABC) co-expressed LYVE-1 (green) suggesting that these cells trans-differentiated into lymphatic vessels in mice. Scale bar= 10㎛. Blue fluorescence indicates DAPI.

Figure-7

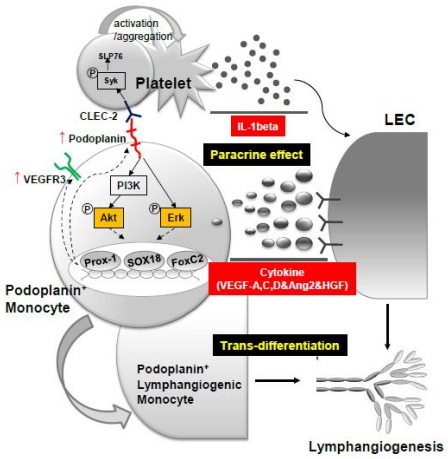

Figure 7. Schematic diagram of lymphangiogenesis by the synergistic interaction between podoplanin-positive monocytes and platelets through podoplanin/CLEC-2 interaction.

When we make hamatosphere by suspension culture of human peripheral blood monocytes under high cell density, there is a huge expansion of podoplanin-positive cells in hematospheres for several days. These cells express lymphatic endothelial cell-specific markers, such as Prox-1, Sox18, FoxC-2, and VEGFR-3. When podoplanin on these monocytes is stimulated with CLEC-2 on platelets, the interaction of CLEC-2 on platelets and podoplanin on monocytes turned on Akt or Erk signaling pathways that provoke two changes on monocytes; (1) the enhanced expression of Prox-1, Sox18, FoxC-2, and VEGFR-3 and the transdifferentiation to

lymphatic endothelial cells, (2) the enhanced secretion of lymphangiogenic cytokines such as VEGF-A,C,D, Angiopoietin-2, and HGF. Meanwhile, the interaction of CLEC-2 on platelets and podoplanin on monocytes stimulates platelets leading to morphologic changes and secretion of lymphangiogenic cytokine, IL-1beta. Overall, these interactions facilitate lymphatic neovascularization and then would healing.

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국문초록

포도플라닌 표지 혈구세포의 림프관내피세포 성질 획득과 혈소판과의 상호작용을 통한 림프관신생에 관한 연구

목적: 최근 연구 보고에서는 마우스 포도플라닌 표지 혈구세포가 림프관 신생에 관련할 수 있다고 밝혔다. 이에 인간 혈액으로부터 포도플라닌 표지 혈구세포를 얻을 수 있는지, 있다면 혈소판과 어떻게 상호작용을 하고 이것이 임상적으로 효과가 있는지에 대해서 밝히고자 한다.

방법과 결과: 인간 말초 혈액 단핵구 세포를 고밀도 초저부착 배양 접시에서 응집 배양 시 세포괴를 만들며, 이를 헤마토스피어라고 명명한다. 5일간 헤마토스피어 배양아래, 포도플라닌 표지 혈구세포는 급격하게 증가하며 림프관 내피세포 특이적인 VEGFR-3 와 림프관 특이 전사 인자를 발현한다. 이후 우리는 포도플라닌 표지 혈구세포와 포도플라닌 수용체 씨렉-2를 발현하고 있는 혈소판과의 잠재적 반응을 관찰했다. 포도플라닌 혈구세포와 혈소판의 공동 배양 후 림프관 내피세포 표지인자가 강하게 발현하는 것을 확인했고, 림프관 특이 사이토카인 또한 증가하는 것을 확인했다. 재조합 씨렉-2는 또한 포도플라닌 표지 단핵구에 Akt 와 Erk 신호전달을 통해 자극시킨다. 마찬가지로 혈소판은 공동 배

양한 단핵구에 의해 자극이 되며, 림프관 특이 사이토카인 IL-1beta가 증가하게 된다. 공동 배양한 배양액은 림프관 내피세포의 이동, 생존능력, 증식을 향상시킨다. 면역 결핍된 마우스 피부 상처에 포도플라닌 표지 단핵구 세포와 혈소판의 국소 주입은 신생림프관 형성을 상당히 증가시키고 상처 치유를 촉진시킨다.

결론: 포도플라닌 표지 단핵구 세포와 혈소판의 공동배양은 포도플라닌/씨렉-2 상호작용을 통해 림프관 신생을 증진시키며, 이는 인간 림프관 질병에 유망한 세포 치료제가 될 것이다.

주요어 : 포도플라닌, 말초혈액 단핵세포, 단핵구, 혈소판, 씨렉-2, 림프관신생

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