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MONOCYTES CAN BE INDUCED TO EXPRESS LYMPHATIC PHENOTYPES

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ABSTRACT

Although it has been recently shown that monocytes can transdifferentiate into blood vascular endothelial cells which are involved in angiogenesis, little attention has been paid to their potential to transdifferentiate into lymphatic endothelial cells. Therefore, we examined this question in our study. We first stimulated monocytes with either fibronectin (FN), VEGF-C, TNF-α, LPS, or IL-3 for 24h. Then we examined the expression of several markers of lymphatic endothelium and found that the monocytes expressed specific lymphatic endothelial markers, LYVE-1, Podoplanin, and Prox-1, but not common endothelial markers vWF or eNOS. Next, monocytes were incubated in endothelial growth medium with FN and VEGF-C for 6d. These monocytes were also found to express LYVE-1, Podoplanin and Prox-1, but not vWF or eNOS. Our results indicate that monocytes in vitro *can be easily induced to present lymphatic phenotypes in an inflammatory environment.*

Keywords: monocyte, transdifferentiation, lymphatic endothelium, RT-PCR, inflammation, immunohistochemistry

Peripheral blood monocytes, an important component of the immune system, participate in many pathophysiological processes by emigrating from blood vessels to local tissues and transforming into macrophages as antigen presenting cells. Previous reports have demonstrated that monocyte-macrophages participate in lymphangiogenesis by secreting VEGF-C, which is to date the most definitive factor for lymphangiogenesis, or by integrating into sprouting lymphatic vessels in tumor progression, inflammation, and immunological rejection *in vivo* (1-3). Recently monocyte-macrophages have also been highlighted due to their plasticity. They have been shown to acquire characteristics of pluripotent stem cells under certain *in vitro* conditions (4,5), and even transdifferentiate into endothelial progenitor cells (EPCs) or endothelial cells in endothelial growth conditions (6-8). These findings called our attention to the question of whether monocytes could transdifferentiate into lymphatic endothelial cells themselves and we undertook the corresponding experiments. Because inflammation is intimately involved in the pathological processes mentioned above, and during inflammation tissue cells secrete a group of factors, including but not limited to FN, VEGF-C, TNF-α, LPS, IL-3, we analyzed the expression of lymphatic endothelial phenotypes in monocytes in response to these factors and whether they could be induced into LECs *in vitro*.

TABLE 1
Primers used for RT-PCR Analysis

	Forward primer	Reverse primer	Product size (bp)
ß-actin	5' CCATCTACGAGGGGTATGCCC3'	5' TCCTTAATGTCACGCACGATTTCC3'	151
Podoplanin	5' GCCAGCCAGAAGATGACACTG3'	5' GAATGCCTGTTA CACTGTTGACAC3'	159
Prox-1	5' CACCTGAGCCACCACCCTTG3'	5' GCATTGCACTTC CCGAATAAGGT3'	136
vWF	5' CTGTGTGGGAATTTTGATGGCATC3'	5' CTGTGTGGGAATTTTGATGGCATC3'	165
eNOS	5' CGGCATCACCAGGAAGAAGAC3'	5' GGTCTCGGAGCCATACAGGAT3'	121

MATERIALS AND METHODS

Monocyte Culture

Monocytes were obtained from peripheral blood mononuclear cells (PBMCs) of healthy male volunteers using a selective attachment procedure as previously described (4) under a protocol approved and supported by the Provincial Foundation. Briefly, PBMCs for this procedure were isolated from buffy coats by Ficoll density-gradient centrifugation (TBD science) and two washes in phosphate-buffered saline (PBS). Cells were suspended at a density of $1x10^6$/ml in DMEM (Gibco) which was supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 g/ml streptomycin, and incubated in plastic bottles at 37°C (5% CO_2) for 6h. After floating cells were removed, adherent cells were rinsed three times with fresh medium and gently detached from the bottle wall by pipetting with medium. These preparations contained 95% monocytes (data not shown) as determined by FAC-Scan (Beckmen-Kulter) flow analysis after immunostaining with R-phycoerythrin (PE)-conjugated mouse anti-human CD14 monoclonal antibody (BioLegend).

To test the effect of the factors on the phenotypes of the monocytes, cells were seeded into 6-well plates pre-coated with 10 µg/ml of fibronectin (Sigma) or non-FN coated with either 50 ng/ml VEGF-C, 100 ng/ml LPS, 50 ng/ml IL-3, or 10 ng/ml TNF-α (all from Sigma) and cultured for 24h. Cells without any FN coating or factor stimulation were used as controls.

To identify whether monocytes can transdifferentiate into lymphatic endothelial cells (LEC), endothelial growth medium EGM-2 (Lonza) with VEGF-C and FN was used. PBMCs were cultured with DMEM supplemented with 10% FBS in FN pre-coated 6-well plates for 6h. Then floating cells were removed, and adherent cells were cultured in EGM-2 with 50 ng/ml of VEGF-C for 6d.

In both sets of experiments, morphological changes of adherent cells were visualized with phase-contrast microscopy (Olympus, Japan), and lymphatic endothelial phenotypes of the cells were tested using RT-PCR and immunohistochemistry. All assays were run in quadruplicate.

RT-PCR

RT-PCR was done both 24h and 6d after culture induction. In brief, total RNA was isolated from the induced cells using Trizol (Takara) according to the manufacturer's instruction. 500 ng of total RNA was reverse transcribed using Prime Script RT (Takara), and cDNA was synthesized at 37°C for 15 min, followed by 85°C for 5s. Initial denaturation was performed at 95°C for 4 min, followed by 35 amplification cycles each consisting of 95°C for 30s, 60°C for 30s, and 72°C for 30s, with a final extension step at 72°C for 2 min. The PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Primers used are listed in *Table 1.*

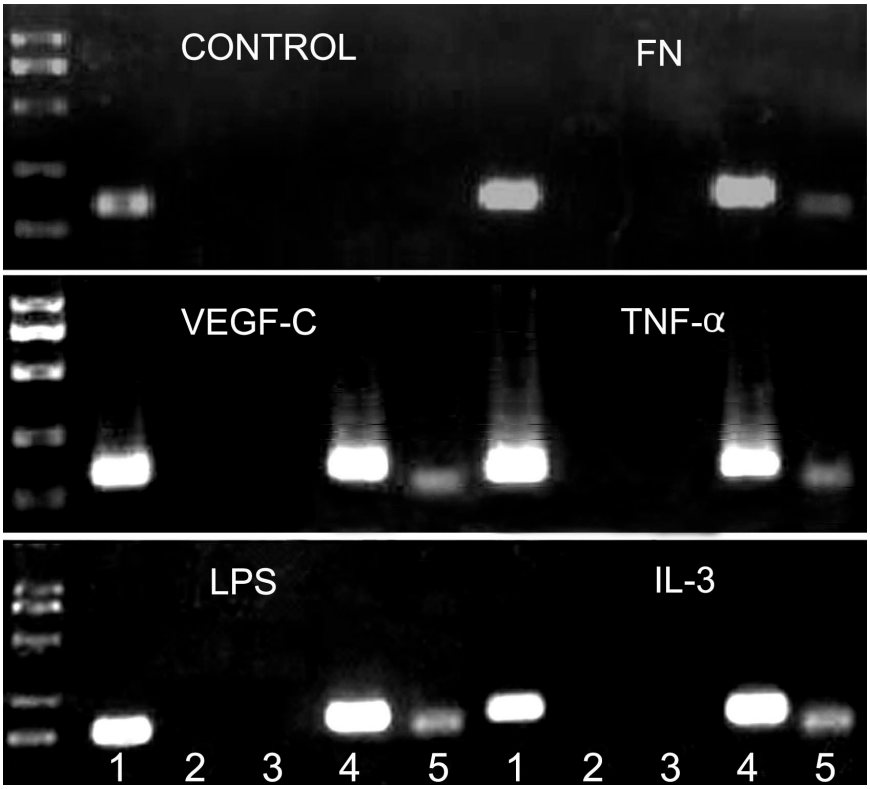

Fig. 1. RT-PCR for induced monocytes. Monocytes were stimulated with VEGF-C, TNF-α, LPS, IL-3, or in FN-coated wells for 24h. Expression of Podoplanin was strongly positive, Prox-1 was weakly positive, and vWF and eNOS were not detected. All controls were negative.1: ß-actin, 2: vWF, 3: eNOS, 4: Podoplanin, 5: Prox-1.

Immunohistochemical Staining

The phenotypes of the adherent cells were examined using Peroxidase Conjugated SABC kits according to manufacturer's instruction. Briefly, adherent cells were fixed in 4% paraformaldehyde and incubated with 1:300 diluted primary antibodies at 4°C overnight. Cells were then washed three times with PBS and incubated with biotin-conjugated goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) for 45 min. The antibody-biotin conjugates were detected with a streptavidin-horseradish peroxidase complex (Santa Cruz) applied for 10 min at room temperature by using 3,3'-diaminobenzidine as the substrate. Mouse mAbs to CD14, CD34, LYVE-1, and Podoplanin, rabbit antibodies to Prox-1, vWF, and eNOS were purchased from Santa Cruz. The primary antibody was omitted for negative controls.

RESULTS

Morphological Transformation

After floating cells were removed at 6h, the adherent cells maintained a round shape. Cultured in FN-coated wells for 24h, one third of the adherent cells became elliptical in shape while all adherent cells from VEGF-C, TNF-α, LPS, IL-3 stimulation groups and the control group (without any stimuli or FN coating) remained round (data not shown). The LEC transdifferentiation induction

TABLE 2
The mRNA Expression Profiles of Monocytes in Experimental Groups

	Podoplanin	Prox-1	vWF	eNOS
Control (24h)	-	-	-	-
FN (24h)	+	+	-	-
VEGF-C (24h)	+	+	-	-
TNF-α (24h)	+	+	-	-
IL-3 (24h)	+	+	-	-
LPS (24h)	+	+	-	-
EGM-2+FN+VEGF-C (6d)	+	+	-	-

experiments demonstrated that the adherent cells transformed into spindle shapes after the 6d incubation.

Transcriptome Analysis

By RT-PCR, Podoplanin was detected after 24h incubation with FN, VEGF-C, LPS, TNF-α, and IL-3. The expression of Prox-1 was comparatively weaker. A similar result was observed in the LEC transdifferentiation induction experiment (data not shown). The mRNA of vWF and eNOS was not detected in any groups and the controls were all negative (*Fig. 1, Table 2*).

Immunohistochemistry Analysis

The primary adherent cells expressed CD14, but not CD34. Following treatment with VEGF-C, TNF-α, LPS, IL-3, or culture in FN-coated wells for 24h, adherent cells were positive for Podoplanin and LYVE-1, while the expression of Prox-1 was comparatively weaker (data not shown), and both vWF and eNOS were negative. In the control group (without any stimuli or FN coating), adherent cells did not express Podoplanin, LYVE-1, Prox-1, vWF, or eNOS (data not shown) throughout the experiment. Following culture in EGM-2 plus VEGF-C and FN-coating for 6d, adherent cells were positive for Podoplanin, LYVE-1, vWF, and eNOS with expression of vWF and eNOS notably weaker than that of Podoplanin and LYVE-1 (*Fig. 2*).

DISCUSSION

Our experiments were useful to investigate the influence of FN, VEGF-C, TNF-α, LPS, and IL-3 on cultured monocytes to induce specific lymphatic markers. Commonly, FN has been used as a reagent for cell adhesion or differentiation. Here, for the first time, we have shown that FN could induce monocytes to express LYVE-1, Podoplanin, and Prox-1. FN activates monocytes by binding to α5β1 integrin on its surface (9), and it may also induce monocytes to express lymphatic markers through the same pathway. VEGF-C is well known for its pivotal role in lymphangiogenesis, and the binding to VEGFR-3 on lymphatic endothelial cells promotes *de novo* formation of lymphatic vessels (10). Monocytes-macrophages have been shown to express VEGFR-3 in inflammatory conditions (11), and when we used VEGF-C as a stimulator to monocytes, we found that VEGF-C could definitely induce monocytes to express lymphatic markers. We also found that IL-3, a pleiotropic factor produced primarily by activated T cells, as well as TNF-α and LPS, two of the most frequently used inflammatory factors, all effectively induced the expression of lymphatic markers in monocytes.

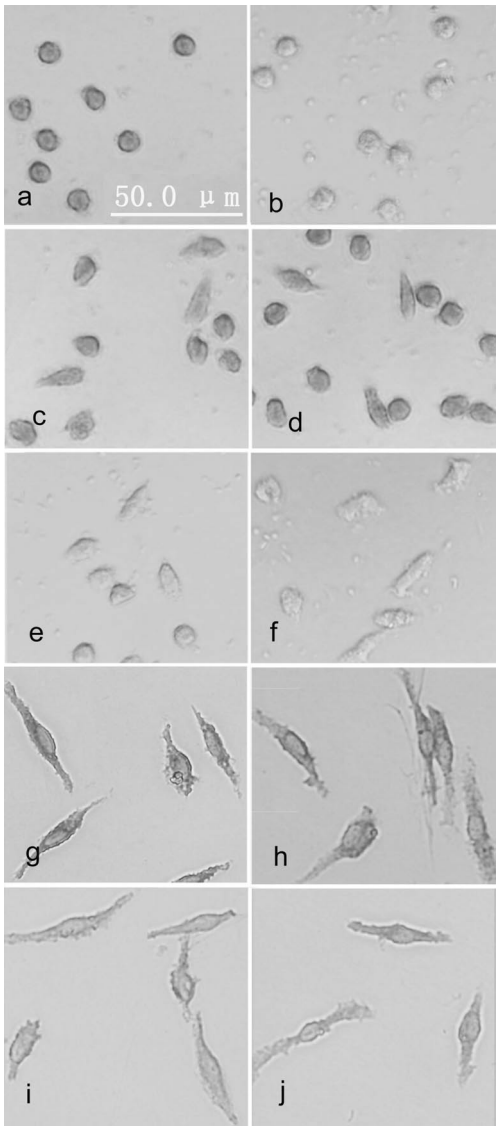

Fig. 2. Monocyte culture immunohistochemistry. After culture for 6h, adherent cells were round in shape and demonstrated CD14 positive (a) and CD34 negative (b) staining. After 24h of culture in FN-coated wells alone, some adherent cells in became elliptical and expressed both LYVE-1 (c) and Podoplanin (d), while vWF (e) and eNOS (f) were negative. Similar phenotypic expressions were obtained in experiments conducted with VEGF-C, LPS, TNF-α, IL-3 as in the FN-coated group (data not shown). After 6 days in the LEC transdifferentiation induction experiment, adherent cells were spindle shaped and demonstrated positive staining for LYVE-1 (g) and Podoplanin (h) with weaker expression of vWF (i) and eNOS (j). 400x

Our study further investigated whether monocytes could transdifferentiate into lymphatic endothelial cells *in vitro*. We cultured monocytes for as long as 6d with EGM-2, a medium containing VEGF, bFGF, EGF, IGF etc. commonly employed to enhance the growth of endothelium, and provided them with both FN and VEGF-C. Most of the monocytes transformed to spindle shapes by day 6 and expressed lymphatic markers on both proteinous and transcriptional levels. However, pan-endothelial markers (vWF and eNOS) proved only weakly expressed by immunocytochemistry and were not detected by RT-PCR, indicating that these cells were not typical vascular endothelial cells.

Monocyte-derived EPCs have attracted extensive interest in the past decade. Some believe that monocytes can differentiate into EPCs under angiogenic conditions, or transdifferentiate into endothelial-like cells that express endothelial markers and participate in the processes of renewal of vascular endothelium or revascularization (8). Nevertheless, the contention that monocytes can be induced into EPCs has been vigorously disputed recently (12). The expression of pan-endothelial markers had been once considered as one of the properties of monocyte-derived EPCs or endothelial cells, and some have argued that this was the result of platelet contamination during experiments (13). In 2009, Haiming Chen et al transcriptionally demonstrated the expression of vWF and VEGFR-2 in monocytes with the simultaneous stimulation of M-CSF and PTP. The expression was enhanced when combined with VEGF, but lacking when the cytokines were used separately (14). However, a previous group cultured monocytes under similar conditions and demonstrated that VEGF caused a large proportion of M-CSF treated monocytes to express a series of endothelial markers (4). Irrespective of the conditions, both articles suggest the possibility for monocytes to transdifferentiate into endothelial cells and

that M-CSF and PTP seemed intimately involved. Since neither of these two factors were employed in this experiment, the weak expressions of vWF and eNOS may likely be due to contamination by platelets.

Although the monocytes failed to express pan-endothelial markers in our *in vitro* study, the lymphatic markers were easily induced. It is still unknown whether these phenotypic monocytes might easily integrate into the wall of lymphatic vessels *in vivo*. This issue needs further investigation to help delineate the cascade of events involved in lymphangiogenesis.

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