Differentiation potential of monocytes into lymphatic endothelial cells **★

Liang Yan-hong[1], Zhang Zhao-lin[1], Tian Hua[1], Wang Chang-ming[2], Wang Shi-kun[1], Li Xin[1], Song Tao[1]

Abstract

BACKGROUND: Previous studies have shown that monocytes can transdifferentiate into vascular endothelial cells under the induction of various factors including vascular endothelial growth factor (VEGF). It remains poorly understood whether monocytes can be induced to transdifferentiate into lymphatic endothelial cells *in vitro*.
OBJECTIVE: To explore the possibility of the transdifferentiation of monocytes into lymphatic endothelial cells under inflammatory condition.
METHODS: Fresh monocytes from peripheral blood were collected by Ficoll density gradient centrifugation and cultured in an endothelial cell medium, followed by incubation in fibronectin-plated well or treated with tumor necrosis factor α for 24 hours, respectively. The expression of specific markers of lymphatic endothelial cells, such as LYVE-1, Podoplanin, Porx-1 and VEGF receptor 3 (VEGFR-3), as well as the endothelial cells markers, such as vWF, endothelial nitric oxide synthase (eNOS) and VEGFR-2, were detected by RT-PCR and immunochemical methods.
RESULTS AND CONCLUSION: Prior to induction, monocytes were positive to LYVE-1, but negative for Podoplanin, Porx-1, and VEGFR-3, vWF, eNOS, as well as VEGFR-2. Following induction, the cultured mononcytes were positive for Podoplanin, Prox-1 and VEGFR-3, but remained negative for vWF, eNOS and VEGFR-2. It suggested that monocytes can be induced to express the markers of lymphatic endothelial cells stimulated by fibronectin or tumor necrosis factor α.

[1]Institute of Anatomy & Histology and Embryology, Medical School of Shandong University, Jinan 250012, Shandong Province, China;
[2]Institute of Anatomy & Histology and Embryology, Basic Medical College of Nanjing University of Traditional Chinese Medicine, Nanjing 210046, Jiangsu Province, China

Liang Yan-hong★, Studying for master's degree, Institute of Anatomy & Histology and Embryology, Medical School of Shandong University, Jinan 250012, Shandong Province, China
kittycat12726@yahoo.com.cn

Correspondence to: Tian Hua, Professor, Institute of Anatomy & Histology and Embryology, Medical School of Shandong University, Jinan 250012. Shandong Province, China
sduth@yahoo.com.cn

Supported by: the Natural Science Foundation of Shandong Province, No. ZR2009CZ014*

Received: 2009-11-04
Accepted: 2010-02-07
(20091104008/WL)

Liang YH, Zhang ZL, Tian H, Wang CM, Wang SK, Li X, Song T. Differentiation potential of monocytes into lymphatic endothelial cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2010;14(10): 1897-1900

[http://www.crter.cn http://en.zglckf.com]

INTRODUCTION

Lymphangiogenesis plays a crucial role in pathophysiological process of tumor, wound and inflammation. Previous studies have shown that lymphangiogenesis generally takes place in two alternative pathways: new lymphatic vessels sprout out from existing vessels under the induction of vascular endothelial growth factor (VEGF)-C[1-2]; or endothelial progenitor cells in peripheral blood migrate to the place of extension and transdifferentiate into lymphatic endothelial cells in which the stimulation of VEGF-C is also involved[3]. Under the condition of tumor, wound or inflammation, monocytes migrate to local tissues and differentiate into macrophages, which perform important functions in the process of lymphangiogenesis by producing VEGF-C[4-6]. Monocytes have been proved to transdifferentiate into vascular endothelial cells under the induction of various factors including VEGF[7-9], but whether they have the ability to become lymphatic endothelial cells is not yet to be shown *in vitro.* Here, several factors or proteins which activate monocytes in inflamed tissues, such as fibronectin and tumor necrosis factor α (TNF-α), were selected to stimulate monocytes *in vitro*. Specific markers of lymphatic endothelial cells LYVE-1[10-11], Podoplanin[12-13], Porx-1[14], VEGF receptor (VEGFR)-3 and common markers of endothelial cells vWF, endothelial nitric oxide synthase (eNOS), and VEGFR-2 were examined. The possibility of monocytes differentiating into lymphatic endothelial cells was explored.

MATERIALS AND METHODS

Design

*In vitro i*nduction, observation experiment.

Time and setting

The experiment was performed at the laboratory of Institute of Anatomy & Histology and Embryology, Medical School of Shandong University, from April to September 2009.

Materials

Human peripheral blood was collected from healthy volunteers.

Reagent and instruments	Sources
Human fibronectin (FN)	Hematech, USA
TNF-α, primary antibody: mouse anti-human LYVE-1 monoclonal antibody, mouse anti-human Podoplanin monoclonal antibody, rabbit anti-human Prox1 monoclonal antibody	Peprotech, UK
Primary Antibody: rabbit anti-human VEGFR-3 monoclonal antibody, rabbit anti-human vWF monoclonal antibody, rabbit anti-human eNOS monoclonal antibody or rabbit anti-human VEGFR-2 monoclonal antibody (ZSGB-BIO, China); Secondary Antibody: FITC-conjugated goat-anti-rabbit or goat-anti-mouse multiclonal antibodies and TRITC-conjugated goat-anti-rabbit multiclonal antibodies	ZSGB-BIO, China
Trizol, Prime Script RT Kit	TakaRa, Japan
Endothelial cell medium (ECM)	Sciencell, USA
Biophotometer	Eppendorf, Germany
PCR Instrument	Whatman Biometra, Germany
Fluorescence microscope (IX70), inverted phase contrast microscope	Olympus, Japan
CO_2 culture incubator	Thermo, USA

Methods

Preparation and culture of mononuclear cells

Peripheral blood mononuclear cells (MNCs) were obtained from healthy donors using method of Ficoll density gradient centrifugation. The mononuclear cell fraction was collected, washed twice with phosphate buffered saline, resuspended in ECM supplemented with 10% fetal bonvine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at a density of 1×10^6/mL and seeded in 24-well plates. The cells were incubated at 37 ℃ with 5% CO_2 for 12 hours. The medium containing floating cells was exchanged with fresh medium and the adherent cells were collected as monocytes and used in the following experiments. The MNCs were divided into 2 groups. One was treated with 10 ng/mL of TNF-α. The other group was seeded in a plate pre-coated with 10 μg/mL of FN. Non-stimulated MNCs seeded in non-FN pre-coated plate were served as control group. The expression of specific markers of lymphatic endothelial cells and the markers of endothelial cells were detected by RT-PCR and immunochemical methods at 24 hours after culture.

RT-PCR

RT-PCR was performed at 24 hours after induction. In brief, total RNA was isolated from the cultured MNCs using Trizol according to the manufacturer's instruction. A total of 500 ng of total RNA was reverse transcribed using Prime Script RT kits and cDNA was synthesized in the condition of 37 ℃ for 15 minutes, followed by 85 ℃ for 5 seconds. Conventional PCR was performed using cDNA together with the PCR master mix using respective primers. The reaction conditions were: 95 ℃ for 30 seconds, 58 ℃ for 30 seconds, 72 ℃ for 30 seconds and a final extension phase of 2 minutes for 38 cycles. The PCR products were electrophoresed on 2% agarose gels, visualized by ethidium bromide staining. Primers used are as follows:

Primers	Sequences
β-actin	Forward: 5'-CCA TCT ACG AGG GGT ATG CCC-3' Reverse: 5'-TCC TTA ATG TCA CGC ACG ATT TCC-3'
LYVE-1	Forward: 5'-TGC TTG CTC TCC TCT TCT TTG G-3' Reverse: 5'-CTT GGA CTC TTG GAC TCT TCT GG-3'
Podo planin	Forward: 5'-GCC AGC CAG AAG ATG ACA CTG-3' Reverse: 5'-GAA TGC CTG TTA CAC TGT TGA CAC-3'
Prox-1	Forward: 5'-CAC CTG AGC CAC CAC CCT TG-3' Reverse: 5'-GCA TTG CAC TTC CCG AAT AAG GT-3'
vWF	Forward: 5'-CTG TGT GGG AAT TTT GAT GGC ATC-3' Reverse: 5'-CTG TGT GGG AAT TTT GAT GGC ATC-3'
eNOS	Forward: 5'-CGG CAT CAC CAG GAA GAA GAC-3' Reverse: 5'-GGT CTC GGA GCC ATA CAG GAT-3'
VEGFR-3	Forward: 5'-AAG TAC ATC AAG GCA CGC ATC-3' Reverse: 5'-GCA GTT CAG GAC CAG CTT CT-3'
VEGFR-2	Forward: 5'-CCG TCA AGG GAA AGA CTA CG-3' Reverse: 5'-CTT TAC CCC AGG ATA TGG AG-3'

Immunocytochemical staining

At 24 hours after culture, lymphatic specific markers and common endothial markers were identified by immunocytochemical method. The cells were stained with mouse anti-human LYVE-1 monoclonal antibody, mouse anti-human Podoplanin monoclonal antibody, rabbit anti-human Prox1 monoclonal antibody, rabbit anti-human VEGFR-3 monoclonal antibody, rabbit anti-human vWF monoclonal antibody, rabbit anti-human eNOS monoclonal antibody or rabbit anti-human VEGFR-2 monoclonal antibody. FITC-conjugated goat-anti-rabbit or goat-anti-mouse multiclonal antibodies and TRITC-conjugated goat-anti-rabbit multiclonal antibodies were utilized as secondary antibody and the cells were immediately observed under an inverted fluorescence microscope.

Main outcome measures

The expression of specific markers of lymphatic endothelial cells such as LYVE-1, Podoplanin, Porx-1and VEGFR-3, and the markers of endothelial cells such as vWF, eNOS and VEGFR-2 were detected using RT-PCR and immunochemical methods.

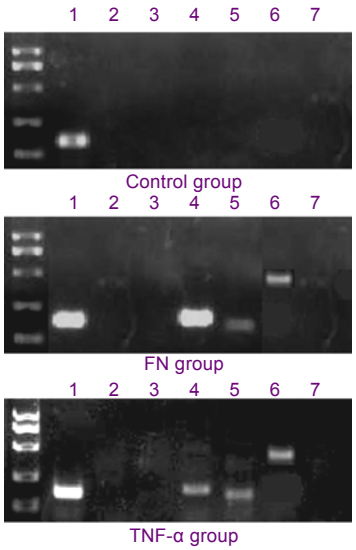
Design, enforcement and evaluation

The experiment was designed by Tian Hua, performed by Liang Yan-hong, Zhang Zhao-lin, Wang Chang-ming, Wang Shi-kun, Li Xin, Song Tao, and evaluated by Tian Hua. All persons were trained regularly. The experiment results were evaluated by blind method.

RESULTS

RT-PCR

The mRNA of Podoplanin, Porx-1, VEGFR-3, vWF, eNOS and VEGFR-2 were negatively expressed in the control group. While, cells of the induction groups were positively expressed Podoplanin, Porx-1 and VEGFR-3. However, mRNA of vWF, eNOS and VEGFR-2 were negatively expressed (Figure 1).

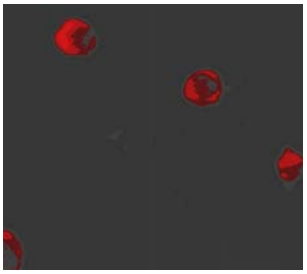


FN: human fibronectin; TNF-α: tumor necrosis factor α; 1: β-actin; 2: vWF; 3: eNOS; 4: Podoplanin; 5: Prox-1; 6: VEGFR-3; 7: VEGFR-2

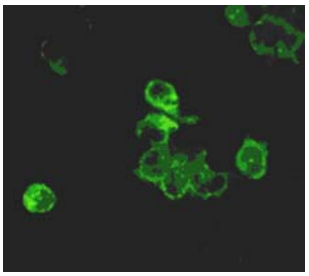
Figure 1 The mRNA expression of monocytes in each group

Immunocytochemical staining

By immunocytochemistry, monocytes of the induction groups were stained positive for Podoplanin, Porx-1 andVEGFR-3, while the expression of vWF, eNOS and VEGFR-2 remained negative. In the control group, the expression of LYVE-1 was positive while the expression of Podoplanin, Porx-1, VEGFR-3, vWF, eNOS and VEGFR-2 were all negative (Figure 2).



a: VEGFR3



b: Podoplanin

Figure 2 Immunofluorescence cytochemistry staining: monocytes expressed VEGFR-3 and Podoplanin after stimulation by tumor necrosis factor α (×400)

DISSCUSSION

Monocytes are an important proportion of cells in peripheral blood, which migrate to local tissues under the condition of tumor, wound or inflammation, and differentiate into macrophages. In these pathological processes, various cells groups including macrophages produce FN, TNF-α and VEGF-C, which may exert certain effects on macrophages transdifferentiating into lymphatic endothelial cells. A study on murine cornea demonstrated that newly formed lymphatic vessels in macrophage infiltrating area were not continuous with limbal existing lymphatic vessels, but were closely related to $CD11b^+$ macrophages in that area[15]. Thus, monocytes may have the potential to transdifferentiate into lymphatic endothelial cells, which is yet to be proved in vitro condition. The monocytes were obtained as described[16]. Firstly, several specific markers of lymphatic endothelial cells on freshly collected monocytes from healthy donors were examined. The expression of LYVE-1 was positive while the expression of Podoplanin, Porx-1 and VEGFR-3 as well as common antigens of endothelial cells vWF, eNOS andVEGFR-2 turned out to be negative. Subsequently, macrophage expression of these markers was tested under different culture conditions. Following 24 hours incubation, Podoplanin, Porx-1 and VEGFR-3 were positive detected by RT-PCR in FN-plated group and in TNF-α treated group. However, the expression of vWF, eNOS and VEGFR-2 remained negative. By immunocytochemistry, the expression of Podoplanin, Porx-1, and VEGR-3 was positive, while vWF, eNOS and VEGFR-2 was negative. According to previous researches, monocytes will transdifferentiate into vascular endothelial cells under the stimulation of growth factors including VEGF[7-9]. Thus, we suppose that the lack of vWF, eNOS and VEGFR-2 was due to insufficient amount of VEGF or inadequate induction period. VEGFR-3 is a lymphatic-specific marker[17-18]. As it is the receptor of VEGF-C which is a lymphatic-specific growth factor, VEGFR-3 plays an important role in the lymphangiogenesis. In tumor or inflamed condition, local macrophages become a crucial resource of VEGF-C[19]. In addition, both VEGF-C and its receptor VEGFR-3 have been reported to be produced and expressed by activated monocytes[20]. In this study, stimulated with FN or TNF-α, monocytes expressed VEGFR-3. The expression of VEGFR-3 is important for monocytes differentiating into lymphatic endothelial cells.

Fibronectin is a high-molecular weight glycoprotein that distributes extensively in extracellular matrix. It is produced by various cells types including fibroblasts, monocytes and tumor cells, and its production increases in inflamed condition. Usually, FN is used as an adhesive in monocyte and neurocyte culturing. In recent years, however, FN is discovered to play a crucial role in migration and differentiation of cells and tissue repairing[21]. We proved that with the induction of FN, monocytes expressed Podoplanin, Porx-1 and VEGFR-3, but the mechanism is yet to be discovered.

TNF-α is one of the most common inflammatory factors produced by various cells types. By inducing monocytes with TNF-α, Podoplanin, Porx-1 and VEGFR-3 were positively expressed. Previous study proved that TNF-α induced macrophages in tumor stroma to produce VEGF-C[22], while TNF-α upregulated the VEGFR-3 expression simultaneously[23]. Therefore, TNF-α inducing the expression of lymphatic endothelial markers may be mediated by VEGFR-3 pathway.

In summary, monocytes expressed lymphatic endothelial markers in a short period of stimulation with TNF-α and FN, while the expression of endothelial common markers was negative. It is supposed that monocytes may completely transdifferentiate into lymphatic endothelial cells with sufficient amount of endothelial growth factors such as VEGF. Considering the scarcely distributed endothelial progenitor cells in peripheral blood, monocytes may be an ideal resource of lymphatic endothelial progenitor cells, because they are more abundant in amount and easy to collect, which is of great value in clinical use.

REFERENCES

[1] Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. 1996;15(2):290-298.

[2] Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science. 1997;276(5317):1423-1425.
[3] Kerjaschki D, Huttary N, Raab I, et al. Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat Med. 2006;12(2):230-234.
[4] Kerjaschki D, Regele HM, Moosberger I, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol. 2004;15(3):603-612.
[5] Schledzewski K, Falkowski M, Moldenhauer G, et al. Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. J Pathol. 2006;209(1):67-77.
[6] Watari K, Nakao S, Fotovati A, et al. Role of macrophages in inflammatory lymphangiogenesis: Enhanced production of vascular endothelial growth factor C and D through NF-kappaB activation. Biochem Biophys Res Commun. 2008;377(3):826-831.
[7] Schmeisser A, Garlichs CD, Zhang H, et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res. 2001;49(3):671-680.
[8] Kuwana M, Okazaki Y, Kodama H, et al. Endothelial differentiation potential of human monocyte-derived multipotential cells. Stem Cells. 2006;24(12):2733-2743.
[9] Rohde E, Malischnik C, Thaler D, et al. Blood monocytes mimic endothelial progenitor cells. Stem Cells. 2006;24(2):357-367.
[10] Banerji S, Ni J, Wang SX, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol. 1999;144(4):789-801.
[11] Prevo R, Banerji S, Ferguson DJ, et al. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem. 2001;276(22):19420-19430.
[12] Breiteneder-Geleff S, Matsui K, Soleiman A, et al. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol. 1997;151(4):1141-1152.
[13] Breiteneder-Geleff S, Soleiman A, Kowalski H, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol. 1999;154(2):385-394.
[14] Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell. 1999;98(6):769-778.
[15] Maruyama K, Ii M, Cursiefen C, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 2005;115(9):2363-2372.
[16] Yong Zh, David G, Eliezer H. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. PNAS. 2003;100(5):2426-2431.
[17] Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995;92(8):3566-3570.
[18] Sinzelle E, Van Huyen JP, Breiteneder-Geleff S, et al. Intrapericardial lymphangioma with podoplanin immunohistochemical characterization of lymphatic endothelial cells. Histopathology. 2000;37(1):93-94.
[19] Cursiefen C, Chen L, Borges LP, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest. 2004;113(7):1040-1050.
[20] Skobe M, Hamberg LM, Hawighorst T, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol. 2001;159(3):893-903.
[21] White ES, Livant DL, Markwart S, et al. Monocyte-fibronectin interactions, via alpha(5)beta(1) integrin, induce expression of CXC chemokine-dependent angiogenic activity. J Immunol. 2001;167(9):5362-5366.
[22] Schoppmann SF, Birner P, Stöckl J, et al. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol. 2002;161(3):947-956.
[23] Hamrah P, Chen L, Cursiefen C, et al. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) on monocytic bone marrow-derived cells in the conjunctiva. Exp Eye Res. 2004;79(4):553-561.

单核细胞向淋巴管内皮细胞诱导分化的潜能*★

梁艳红[1]，张肇林[1]，田　铮[1]，王长明[2]，王世坤[1]，李　鑫[1]，宋　涛[1]（[1]山东大学医学院解剖与组织胚胎学研究所，山东省济南市　250012；[2]南京中医药大学基础医学院解剖与组织胚胎学教研室，江苏省南京市　210046）

梁艳红★，女，1981 年生，山东省莱阳市人，汉族，山东大学在读硕士，主要从事淋巴管再生的研究。

通讯作者：田　铮，教授，山东大学医学院解剖与组织胚胎学研究所，山东省济南市 250012

摘要

背景：有研究报道，在包括血管内皮生长因子在内的多种因子诱导下，单核细胞能够转化成血管内皮细胞；而在体外条件下，单核细胞能否转化成淋巴管内皮细胞，至今未见报道。

目的：观察单核细胞在炎症环境中转分化成淋巴管内皮细胞的可能性。

方法：采用 Ficoll 密度梯度离心法分离成人新鲜外周血单核细胞，用细胞培养基培养，然后分别用纤维连接蛋白、肿瘤坏死因子 α 诱导 24 h。用 RT-PCR 和免疫细胞化学方法检测单核细胞对淋巴管内皮特异性标志物 LYVE-1、Podoplanin、Prox1、血管内皮生长因子受体 3 以及内皮细胞抗原 vWF、内皮型一氧化氮合酶和血管内皮生长因子受体 2 的基因和蛋白表达。

结果与结论：单核细胞在诱导之前，对 LYVE-1 表达阳性，对 Podoplanin、Prox1、血管内皮生长因子受体 3、vWF、内皮型一氧化氮合酶和血管内皮生长因子受体 2 表达均为阴性；经上述因子刺激后，单核细胞对 Podoplanin、Prox-1、血管内皮生长因子受体 3 表达阳性，vWF、内皮型一氧化氮合酶和血管内皮生长因子受体 2 表达仍呈阴性。提示纤维连接蛋白、肿瘤坏死因子 α 均能有效刺激单核细胞表达淋巴管内皮标志物。

关键词：单核细胞；转分化；淋巴管内皮细胞；体外培养；诱导

doi:10.3969/j.issn.1673-8225.2010.10.040

中图分类号: R394.2　文献标识码: A

文章编号: 1673-8225(2010)10-01897-04

梁艳红，张肇林，田铮，王长明，王世坤，李鑫，宋涛.单核细胞向淋巴管内皮细胞诱导分化的潜能[J].中国组织工程研究与临床康复, 2010,14(10):1897-1900.

[http://www.crter.org　http://cn.zglckf.com]

(Edited by Zhao LJ/Wang L)

来自本文课题的更多信息--

基金资助：山东省自然科学基金资助项目(ZR2009CZ014)。

利益冲突：无其他利益冲突。