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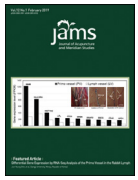

Research Article

Expression of Genes in Primo Vasculature Floating in Lymphatic Endothelium Under Lipopolysaccharide and Acupuncture Electric Stimulation

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lymph vessel;
primo vessel

Abstract
It is known that the primo vascular system (PVS) includes the primo nodes and vessels. However, the relevant genes in the PVS system for both pathologic and physiologic condition are poorly understood. Here, we first examined the gene expression in primo vessels (PVs) floating in lymphatic endothelium by isolation of PVS and lymphatic vessels (LVs) containing PVS. To investigate therapeutic effects, both PVs and LVs containing PVS were isolated after lipopolysaccharide injection and acupuncture electric stimulation at two acupoints Joksamni (ST36) and Hapgok (LI04) following lipopolysaccharide injection. We used reverse transcriptase—polymerase chain reaction to examine expression of lymphatic endothelial cell markers and inflammatory related genes. We found that lymphatic endothelial cell markers such as *fms-related tyrosine kinase 4 (Flt4)*, *lymphatic vessel endothelial receptor (Lyve-1)*, *prospero homeobox protein 1*

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(Prox-1), and *podoplanin (Pdpn)* were highly expressed in PV compared to that of lymphatic endothelium, suggesting pivotal roles of PV in LV under inflammation. Furthermore, lymphatic-related genes including metal-response element-binding transcription factor 2 (*Mtf2)*, *h*ypoxia inducible factor (*Hif1a)*, angiotensin II type 1 receptor (*Agtr1)*, and angiotensin II type 2 receptor (*Agtr2*) were also overall increased in PV, and remarkably increased and these genes except peroxisome proliferator—activated receptor gamma (*Pparg)* after acupuncture electric stimulation in two acupoints implying central role of PV by gene activation.

1. Introduction

Since primo vascular system (PVS) was discovered by Kim in 1962, many articles have been published stating PVS as a circulatory system containing corpuscle and ducts [1—3]. Primo vasculature refers to the meridians in oriental medicine, which are considered to be networks of the body [1]. This PVS, also known as the third circulatory vessel, is one of the important vascular systems aiming at the homeostasis and restoration by inducing the changes and stimulating the state of human body when exposed to the disease [4,5]. Until now, it has been found that there is a very small vessel floating in the lymphatic vessels (LVs) anatomically [6,7]. However, it is not known whether the small primo vessel focuses on any of the functions of lymphatic system. The LV is a tube that plays an important role in absorbing lymphocytes and fats in human body, maintaining body fluid homeostasis, and examining immunity [8]. There are various types of lymph vessels, ranging from small lymphatic capillaries to large LV [9].

Although existence of primo vasculature is revealed in many species including mouse, rat, and rabbits as well as humans, the biologic role of primo vasculature including genes expression and proteins has not yet been investigated [10]. Especially, the experimental results for the transcriptional action by messenger ribonucleic acid (mRNA), which is required to accomplish biological action, need to be studied urgently in PVS biology. Emerging data suggest that pathophysiologic conditions such as tumors can progress into inflamed lymphatic endothelial cells (LECs) and can lead to expanded primo vessels (PVs) in lymphatic endothelium [11].

The expressed genes such as *fms-related tyrosine kinase 4 (Flt4)* [12], *prospero homeobox protein 1 (Prox-1* [13]*)*, *lymphatic vessel endothelial receptor 1 (Lyve-1)* [14], and *podoplanin (Pdpn)* [15] are typical lymphatic markers. In the case of mature LV, markers for hematopoietic stem cells and CD34 are not expressed. It is known that LV is activated by lipopolysaccharide (LPS) or conconavalin inflammation, but the changes in gene expression of PV are unknown [16]. In addition, Joksamni (ST36) and Hapgok (LI04) are known as major two acupoints for treatment in oriental medicine [17,18]. It has been proved that the treatment effect is stimulated through meridian system, but it is also unknown what kind of genetic material is expressed and changed.

This research studied the expression of target genes by measuring from each tissue for two different PVs and LVs. The purpose of this study was to investigate how the expression patterns of target genes are changed by LPS induction of inflammation and acupuncture electric stimulation (AES) at two acupoints ST36 and LI04.

2. Materials and methods

2.1. Sample preparation

The New Zealand female rabbits were purchased from the Daehan Biolink company, and all animal protocols were approved by the Institutional Animal Care and Use Committee of the Sangji University. Rabbits were kept in a room with a constant temperature of 23°C and 60% relative humidity, and alternate light and darkness was maintained for 12 hours each. Experimental animals were allowed to drink water and feed freely without any restriction, so that they maintained optimal condition. Anesthetic (2 mL) mixture containing zoletil and rumpun was injected into the muscle of the rabbits used in the dissection experiment. All anatomical procedures were performed in a general anesthetic environment [19,20]. In this study, we divided rabbits into two experimental groups treated with LPS injection and electric stimulation at two acupoints ST36 and LI04 after LPS injection [21,22]. A total of 10 samples were taken from the experimental groups.

2.2. Procedure of AES after LPS treatment

The device used for AES in the rabbit after LPS injection into the lymph node was the low-frequency electric stimulator (GP-302N) produced by Goodpl Co., Ltd, the medical equipment company in Wonju, Republic of Korea. Two representative meridian points for experimental rabbits were Joksamni (ST36) and Hapgok (LI04), and AES was performed by connecting the instrument to both terminals clipped with needles of two acupoints [19]. The GP-302N has built-in switches that control low frequency waveform, intensity, operation time, low frequency generation confirmation frequency, low frequency current frequency, CPU-controlled automatic program function, and touch panel application and safety function. Procedure of AES is classified into six steps as shown in Table 1.

First, in step 1, acupuncture was performed on the right side of ST36 and on the left side of LI04. The average resistance value between two acupuncture terminals was 1.226 MΩ. The applied stimulation time and the frequency of square waveform for each step were 90 second and 66.7 kHz, and the voltage and the current intensity were

Table 1 The classification of six steps by using AES of model GP-302N. The terminals used for electrical stimulation are two acupoints ST36 and LI04. Applied time, frequency, peak voltage, and current of pulse wave form of AES for each step are same. The total applied time of AES is 540 s.

Step	ST 36		LI04		Acupuncture position*	Electric stimulation condition			
	Right	Left	Right	Left		Time	Frequency	Voltage	Current
1	O			O		90 s	66.7 kHz	33.6 mV	27.4 mA
2		O	O			90 s	66.7 kHz	33.6 mV	27.4 mA
3	O		O			90 s	66.7 kHz	33.6 mV	27.4 mA
4		O		O		90 s	66.7 kHz	33.6 mV	27.4 mA
5			O	O		90 s	66.7 kHz	33.6 mV	27.4 mA
6	O	O				90 s	66.7 kHz	33.6 mV	27.4 mA
Total						540 s			

* The red dotted circle line indicates the position of two acupoints (ST36, LI04) in the front side of rabbit.

33.6 mV and 27.4 mA, respectively. In step 2, acupuncture was performed on the left side of ST36 and the right side of LI04; in step 3, it was performed on the right of ST36 and the right of LI04; in step 4, it was performed on the left of ST36 and the left of LI04; in step 5, it was performed on the right of LI04 and the left of LI04; in step 6, it was performed on the right of ST36 and the left of ST36. The total time of AES was 540 s during the six steps.

2.3. Experimental groups and quantitative reverse transcription–polymerase chain reaction

The following four experimental groups were compared in this study. The first experimental group is a sample containing only PV [Primo only (LPS +, ACUP −), assigned as Group 1]. The second experimental group is a sample [Lymph + Primo (LPS +, ACUP −), assigned as Group 2] in which a PV and a LV are mixed after injection of LPS. The third experimental group is a sample containing only PV [(LPS +, ACUP +), assigned as Group 3], which contains only the sample obtained after the injection of LPS and treatment with electric stimulation therapy at two acupoints ST36 and LI04 in the inflammation-induced condition. The fourth experimental group is a sample [Lymph + Primo (LPS +, ACUP +), assigned as Group 4] mixed with PV and LV obtained after treatment with LPS and inflammation-inducible state by using electric stimulation therapy at two acupoints ST36 and LI04. To perform polymerase chain reaction (PCR), 10 samples (the 6 LV containing PV and 4 pure PV) were used in present study. The relative mRNA expression of target genes was calculated using the comparative *CT* method. All target gene expression was normalized to *Gapdh* expression in multiplexed reactions performed in duplicate. Differences in *CT* values were calculated for each target mRNA by subtracting the mean value of *Gapdh* expression (relative expression $= 2^{-\Delta CT}$),

and two independent reactions were performed. Information regarding the primer sets (Bioneers, Korea) used in this study is provided in Table 2.

2.4. Statistical analysis

All results in this study were expressed as standard value and standard error of mean. Reverse transcriptase polymerase chain reaction (RT-PCR) data were analyzed by using GraphPad Prism, version 4, software (La Jolla, Calif., USA) and considered statistically significant at $p < 0.05$ level [23]. Data for all samples were compared by Mann–Whitney U test.

3. Results

3.1. Observation and extraction of PV of rabbit under LPS treatment

This study completes the basis for this invention by examining the genes in the primo vasculature for the first time. Although PV in purity was a nonvisible vessel, observation is possible through the spread of blue reagents within 20 minutes. PV is carefully collected using tweezers. First, all the organs in the bladder were sideways after excision of the outermost skin along the median line from the abdomen of the rabbit to the symphysis pubis and to the episternum. We injected LPS into the lymph node by looking for a lymphatic bundle near the vein of the abdomen, as shown in Fig. 1A. The amount of LPS injected into the lymph node shown in Fig. 1B was 200 μg/kg [21]. In order to observe the PV in LV, alcian blue (AB) solution was prepared from 0.1 g AB in 10 mL of phosphate-buffered saline (pH 7.4) and was filtered by using a 0.2 um membrane filter with a syringe. AB solution, preheated to 37°C in a water bath, was injected into lymph bundles in part of vena cava. We waited for 5 minutes after LPS treatment, and then we injected AB stain, as shown in Fig. 1C. The AB staining solution was allowed to flow along with the lymph fluid flowing through the LV for 5 minutes, and we were able to observe and extract the PV inside the LV, as shown in Fig. 1D–F. Fig. 1D showed PV inside LV attached organs stained by AB blue. Fig. 1E and F showed the isolated PV inside of LV and the isolated PV from LV, respectively. The PV as a strand like microtubular PVS stained with AB is floating inside a LV. We already identified PVS which has rod-shaped nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride in a previous report [22].

Table 2 Primers and probes for quantitative RT-PCR.

Genes		Primers and probes (5′–3′)
Rabbit *Gapdh*	Forward	ggaagctggtcatcaacggg
	Reverse	ggggctgagatgatgaccct
Rabbit *Flt4*	Forward	gctgctcggaacatcttgct
	Reverse	cgaaggaccacacgtcactg
Rabbit *Lyve-1*	Forward	aggttgaagcggcatggaag
	Reverse	cacccaacccattcttcccg
Rabbit *Pdpn*	Forward	gtccatggagaaagcgggtg
	Reverse	ccttcccgacattttccgca
Rabbit *Prox-1*	Forward	ggccgagaccttgaaacagg
	Reverse	ctgggggatctggagaggtg
Rabbit *Mtf2*	Forward	aactgctgagccacctttgg
	Reverse	ggaagcacccgtgaaatcca
Rabbit *Hif1a*	Forward	gcagactcaggggcaagaac
	Reverse	tggtggtgatgttgtggcac
Rabbit *Pdgfd*	Forward	ccggcgagatgagagcaatc
	Reverse	ccacgtcagaagcaggttcc
Rabbit *Agtr1*	Forward	ggccctcaagaaggcttacg
	Reverse	ctgcagtcgtgaatgacccc
Rabbit *Agtr2*	Forward	cctggatgctctgacctgga
	Reverse	ggagcttctgttggaaccgg
Rabbit *Pparg*	Forward	tcggagggacaaggcttcat
	Reverse	gcttcacattcagcaggcct

RT-PCR = reverse transcriptase-polymerase chain reaction.

3.2. Analysis of gene expression before and after electric stimulation for dose-induced rabbit injection of LPS

Flt4, *Prox-1*, *Lyve-1*, *and Pdpn* are known well as markers for LEC. Hypoxia inducible factor (*Hif)1a*, metal-response element-binding transcription factor 2 (*Mtf2*) and related genes such as angiotensin II type 1 receptor (*Agtr1*), *Agtr2*, platelet-derived growth factors D (*Pdgfd*), and peroxisome proliferator–activated receptor gamma (*Pparg*), which are known to be expressed in the LV of rabbit, were investigated. *Flt4* is a typical marker gene in the lymphatic system, and mouse die at mid-gestation stage due to cardiovascular defects [12]. It plays a role in the development of lymph vessels and lymphangiogenesis and sinusoidal vessels that are involved in cancer or inflammation [16,24]. We found that expression of *Flt4* was overall increased in PV (2.5-fold), suggesting relevant role of PV in lymphatic function via *Flt4*. *Flt4* was sustained by acupoint stimulation and was decreased in PV, then was decreased in Group 4 (in PV, 2.4-fold vs. Group 4; in LV, 3.1-fold vs. Group 4). Even no significant difference was detected in each group; enriched *Flt4* in PV let us expect diverse roles for primo vasculature. Especially, the primary role of mitigation controlled by the primo vessel will be further investigated in disease when the acupoint function of the antiinflammatory function as described in oriental medicine was given to two acupoints ST36 and LI04.

Pdpn is a specific gene for lymphatic endothelium, and *Pdpn* knockout mouse die due to lymphatic malformation. In addition, *Pdpn* bone marrow–derived progenitor cells can function as a lymphatic endothelial progenitor cells [25,26]. It is used as a biomarker for head and neck cancer in clinical practice [27]. The expression level of *Pdpn* in PV was increased 1.8-fold compared to the level in the LV including PV. Noteworthy is that *Pdpn* in PV was significantly increased 6.1- and 375-fold in the Group 4 and Group 3, respectively, after the AES at two acupoints. Increased *Pdpn* in LV may provide clinical significance, especially inflammation and tumor metastasis [25]. It seems to be closely relevant to therapeutic targeting of *Pdpn* in PV.

PCR analysis demonstrated augmented expression of *Prox-1* (1.5-fold) and *Lyve-1* (1.4-fold) in the PV compared

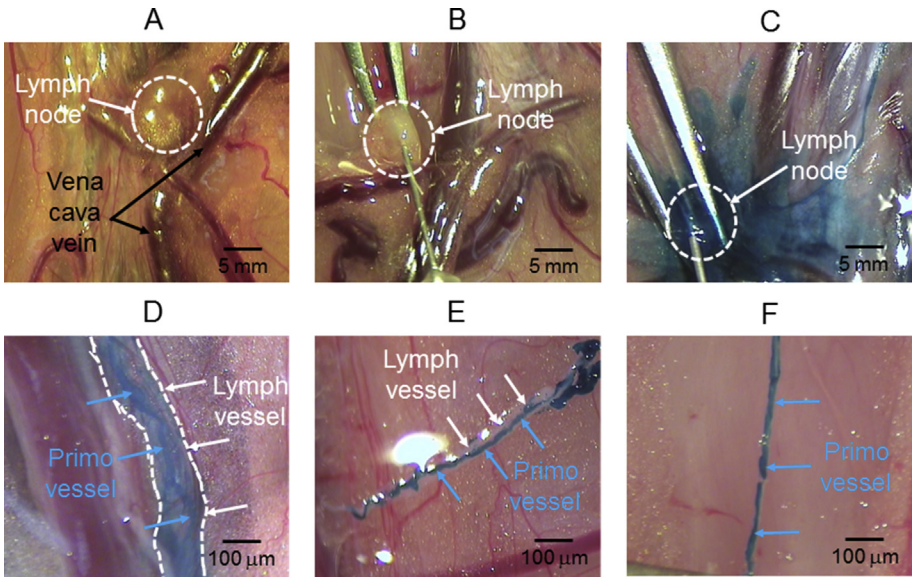

Figure 1 Images of lymph node in the vena cava of a rabbit. (A) Before LPS injection to lymph node. (B) After LPS injection to lymph node. Images of staining lymph node. (C) After AB injection. (D) Floating PV inside a LV. (E) Image of an isolating PV from LV. (F) Image of an isolated PV. White dot circle lines indicate lymph node and white and sky color arrows indicate LV and PV, respectively. AB = alcian blue; LPS = lipopolysaccharide; LV = lymphatic vessel; PV = primo vessel.

with the LV including PV under LPS treatment. *Prox-1* is a central transcription factor for LEC [13,28] and *Lyve-1*, also shown in Fig. 2, is also a typical marker for LEC [14]. Both *Prox-1* and *Lyve-1* also showed higher expression in the PV than in the LV. However, their expressions were significantly reduced when the AES was applied to two acupoints ST36 and LI04 after inflammation (in *Prox-1*; 2.8-fold in Group 3, 2.0-fold in Group 4, in *Lyve-1*; 2.2-fold in Group 3, 1.8-fold in Group 4, vs. Group 1). It suggests inactivation of these genes by acupuncture. Among 4 genes as representative markers for LEC, all genes except *Flt-4* displayed significant difference in PV in terms of AES (Fig. 2).

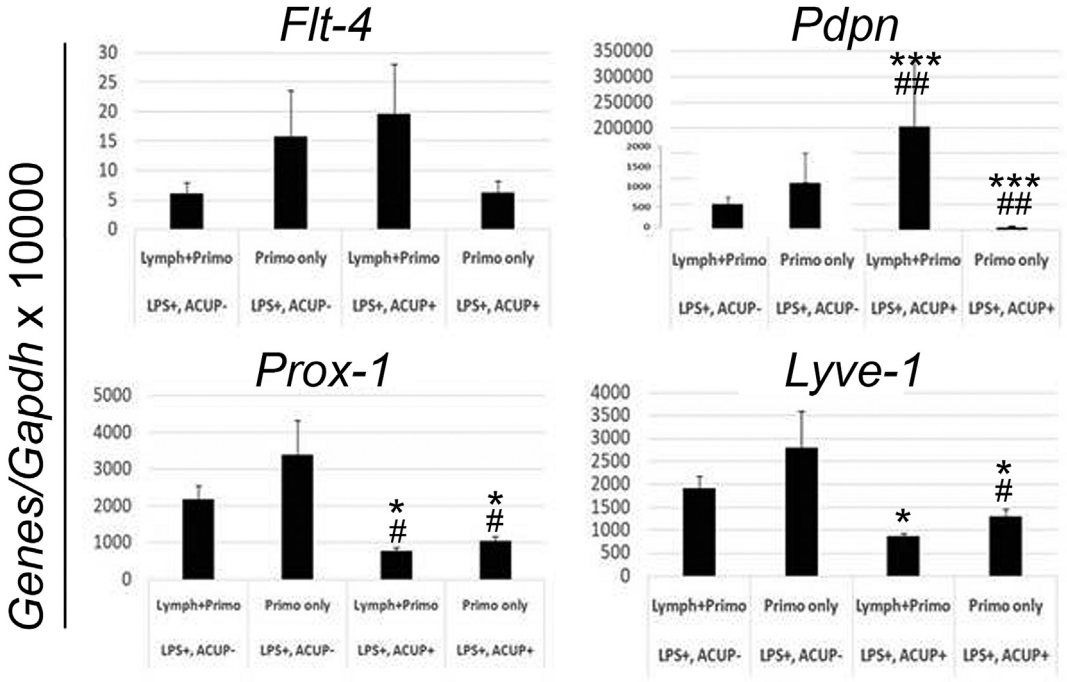

Figure 2 Expression of LEC markers in LV including PV, PV under inflammation was revealed and both LV and PV in acupoint stimulation after LPS was also changed their gene expression. Gene expressions for LV and PV by LPS (+) treatment and before (−) and after (+) AES at two acupoints ST36 and LI04. Samples were harvested and subjected to qRT-PCR. Each value is the average of duplicated experiments (at least 2 per experiment). * and # indicate significant difference versus Group 1 (LV including PV under LPS) and Group 2 (only PV under LPS) (* and $^{\#}p < 0.05$, *** and $^{\#\#\#}p < 0.001$). LEC = lymphatic endothelial cell; LPS = lipopolysaccharide; LV = lymphatic vessel; PV = primo vessel; qRT-PCR = quantitative reverse transcriptase—polymerase chain reaction.

We first found that the expression of genes for LEC is rapidly reduced in the PV than in the LV, suggesting important role of PV in therapeutic stimulation.

Next, we examined several more genes for LEC or inflammation-related marker such *as Mtf2*, *Hif1a*, *Pdgfd*, *Agtr1*, *Agtr2*, and *Pparg*. PCR data for gene expression were depicted in Fig. 3. Rare data for *Mtf2* existed in lymphatic vessel. Although *Mtf2* gene was not revealed in the lymphatic vessels, our data clearly showed the high expression of *Mtf2* in LV (13.5-fold) and PV (7.2-fold) compared to *that of Flt4*, representative marker. Also, significant difference was detected in PV and LV by AES (in LV, 475.7-fold vs. Group 3; in PV, 7.9-fold in Group 4). It is first time *Mtf2* was detected in LV and PV, implying role as an emerging marker in LV.

Hif1a is regarded as the major transcription factor which can control cellular and developmental reaction to hypoxia. It is known that upregulation of *Hif1a* can regenerate damaged tissues having repair response [29]. It has also been shown that the function of *Hifla* in angiogenesis and cancerous environments has already been elucidated, and lymph vessels are also regulated by *Hifla* [30]. Our data in Fig. 3 showed *Hif1a* was remarkably increased with statistical significance in acupoint stimulation (in LV, 162.5-fold vs. Group 3; in PV, 62.3-fold vs. Group 4). It strongly suggests that acupoint stimulation may induce therapeutic effects in LV, especially, PV. PCR data continuously provided the importance of PV in LV. Because *Hif1a* is closely involved in erythropoiesis, we further examined *Hif1a* function in primo vasculature under disease condition.

Similar with *Hif1a*, *gene expression of Pdgfd* significantly increased in groups 3 and 4 compared to that of groups 1 and 2 (in LV, 32.0-fold vs. Group 3; in PV, 32.1-fold vs. Group 4). *Pdgfd* is known as important factor for

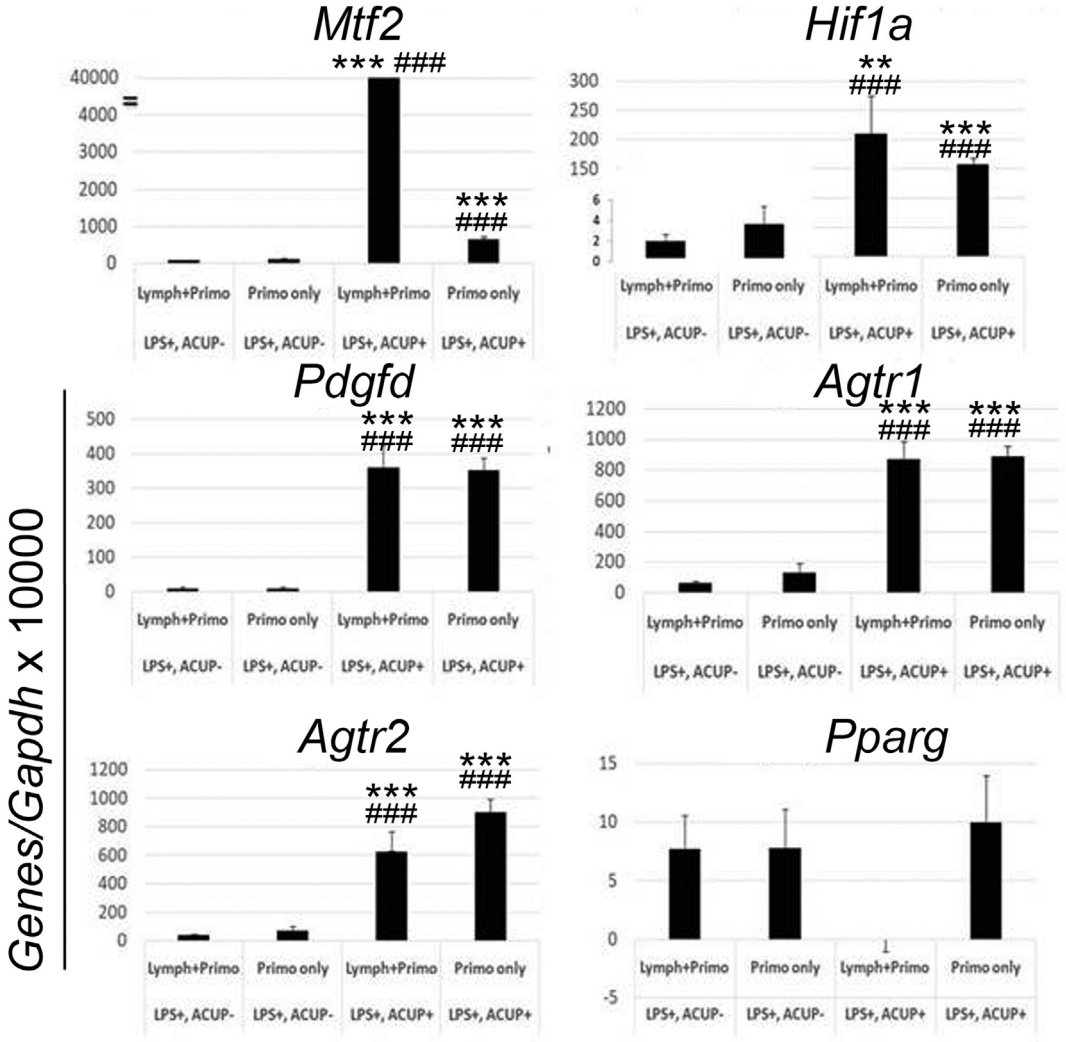

Figure 3 Expression of LEC markers in LV including PV, PV under inflammation was displayed, and both LV and PV in AES after LPS was also changed their gene expression. Gene expressions for LV and PV by LPS (+) treatment and before (−) and after (+) AES at two acupoints ST36 and LI04. Samples were harvested and subjected to qRT-PCR. Each value is the average of duplicated experiments (at least 2 per experiment). * and # indicate significant difference versus Group 1 (LV including PV under LPS) and Group 2 (only PV under LPS) (* and $^{\#}p < 0.05$, ** and $^{\#\#}p < 0.01$, *** and $^{\#\#\#}p < 0.001$). AES = acupuncture electric stimulation; LEC - = lymphatic endothelial cell; LPS = lipopolysaccharide; LV = lymphatic vessel; PV = primo vessel; qRT-PCR = quantitative reverse transcriptase—polymerase chain reaction.

angiogenesis, but the relevance to the lymph vessels is not well known [31]. In the present data, there is no difference in gene expression both in inflammatory LV and PV. *Agtr 1* and 2 were also displayed with similar pattern with *Hif1a* and *Pdgfd*. LEC-related genes were highly increased in terms of acupoint electric stimulation in LV and PV (in *Agtr1*, in LV, 13.5-fold vs. Group 3; in PV, 6.7-fold vs. Group 4; in *Agtr2*, in LV, 14.1-fold vs. Group 3; in PV, 12.6-fold vs. Group 4).

Agtr1 and 2 are involved in contracting blood vessels to induce hypertension, to inhibit the absorption of water, and to discharge them out of the body [32,33]. These genes have recently been shown to be associated with the LV; however, no data in PV existed. Both genes showed a slightly higher expression in primo without significance, showing no specificity for PV. So far, investigated genes were displayed in LV as well as PV. To confirm high expression for these genes in PV with significance, more samples should be collected in separated PV only and LV containing PV. Although no significant difference was detected between LV and PV in LPS treatment, we found that most genes were unarguably increased in LV and PV by acupoint stimulation with statistical significance (Figs. 2 and 3). Meanwhile, *Pparg* was revealed no difference in gene expression, regardless of group. No expression in Group 3, LV including PV after LPS under acupoint stimulation group was detected. *Pparg* was sustained in only PV of Group 4. This data led us to question whether Pparg can modulate in PV but not LV under therapeutic condition and further study will be performed based on present data. *Pparg* is expressed in adipose tissue and macrophage and functions to participate in fatty acid storage and glucose metabolism [34,35]. This result showed possibility of *Pparg* as an optimal candidate in the PV rather than in the LV after acupuncture.

Collectively, our data showed that the function of primo vasculature can overwhelmingly replace the previously known role of the lymph vessel or the possibility of functioning as a marker gene that represents PV. From all the above results, we found that gene expression for LEC marker in primo vasculature and their expression can increase by AES. Based on this result, we will further investigate the functional role of PVS in pathophysiologic condition.

4. Conclusions

We first examined gene expression in both isolated PV and LV containing PV under LPS treatment and AES. This study revealed that LEC-related genes or LEC markers are relatively enriched in PV rather than LV. These genes were significantly increased by AES, which can mimic PV stimulating therapy. It suggests that LEC-related genes in PV are correlated with disease condition with gene activation, and this property can be harnessed for the development of a biomarker for monitoring pathophysiologic conditions such as inflammation and therapeutic progression. Collectively, our data suggest that expression of LEC-related genes or LEC markers in PV depend on AES and raise the expectation of advanced therapeutic strategies involving PV manipulation in pathophysiologic condition.

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