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Adiponectin promotes endothelial cell differentiation from human peripheral CD14$^+$ monocytes *in vitro*

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Abstract

Adiponectin was revealed to have anti-atherogenic and anti-inflammatory properties and has been recently found to stimulate angiogenesis *in vivo* and *in vitro*. However, the role of adiponectin in endothelial differentiation remains unclear. The objective of this study was to investigate whether adiponectin can promote peripheral CD14$^+$ monocytes differentiation into endothelial cells (ECs). Human peripheral blood CD14$^+$ monocytes were cultured with or without adiponectin (10 μg/ml) for 10 days. Adiponectin significantly promoted EC morphology formation from CD14$^+$ monocytes. By flow cytometery analysis, cells treated with adiponection substantially increased mean fluorescence intensity of vascular endothelial growth factor receptor-2 (VEGFR-2) and endothelial nitric oxide synthase (eNOS), two specific endothelial markers, by 49.2 % and 53.9 %, respectively, as compared to control cells. By real time PCR analysis, the mRNA level of eNOS in adiponectin-treated cells was also increased by 31.9 % of that of the control cells. However, the mRNA levels of calponin and SMMHC, two specific SMC markers, in adiponectin-treated cells were decreased by 81.1 % and 79.7 % of that of the control cells, respectively. These data demonstrated that adiponectin could promote endothelial differentiation from peripheral blood CD14$^+$ monocytes by morphology change, upregulation of EC markers and downregulation of SMC markers. Adiponectin-promoted EC differentiation may contribute to vascular healing and angiogenesis.

Keywords: adiponectin • CD14$^+$ monocyte • endothelial cell • differentiation • VEGF receptor-2 • endothelial nitric oxide synthase • angiogenesis • obesity • adipokine

Introduction

Adiponectin, an adipocyte-specific adipokine, has been recently revealed to have anti-atherogenic and anti-inflammatory properties. Decreased plasma adiponectin levels are observed in patients and animal models with obesity, coronary artery disease and type 2 diabetes [1–6]. Plasma adiponectin concentrations are reported to be increased following weight loss and treatment of coronary artery diseases [5, 7]. Overexpression of adiponectin reduces atherosclerotic lesions in mouse models [8], whereas adiponectin-deficient mice exhibit excessive intimal response to vascular injury [9, 10] and diet-induced insulin resistance [11, 12].

More recently, adiponectin was found to stimulate angiogenesis *in vivo* and *in vitro*. Adiponectin promoted the capillary-like structure formation

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from human umbilical vein endothelial cells (HUVECs) *in vitro* and functioned as a chemoattractant for HUVECs in migration assays. Moreover, adiponectin stimulated blood vessel growth in mouse Matrigel plug implantation and rabbit corneal models of angiogenesis [13]. In addition, adiponectin stimulated angiogenesis in response to tissue ischemia [14]. Meanwhile, adiponectin dose-dependently suppressed apoptosis and caspase-3 activity in HUVECs [15]. Angiotensin II-induced apoptosis in human endothelial cells (ECs) was inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase (eNOS) and heat shock protein 90 (HSP90) [16].

However, the roles and mechanisms of adiponectin in endothelial differentiation, which is involved in angiogenesis, neovascularization, and vascular healing, are largely unknown. The mobilization and differentiation of endothelial progenitor cells (EPCs) has been recently shown to be important in the process of adult neovascularization [17–21]. EPCs contribute as much as 25 % of ECs in newly formed blood vessels [22]. The number and migratory activity of circulating EPCs have also been shown to inversely correlate with risk factors for coronary artery disease [23] and to serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk [24]. C reactive protein, a key predictor as well as a mediator of atherosclerosis and coronary artery disease, inhibits EPC differentiation, survival and function [25]. $CD14^+$ monocytes are abundant in human peripheral blood and have been shown to have strong potential to differentiate into ECs [26]. Hence, $CD14^+$ monocytes from human peripheral blood have been included in the candidates of EPCs. Since adiponectin was revealed to play a protective role in the cardiovascular system and to stimulate angiogenesis, we hypothesized that adiponectin may enhance the differentiation of $CD14^+$ monocytes into ECs, thereby favoring neovascularization and vascular healing. Specifically, we examined the effect of adiponectin on morphological change as well as gene expression of EC markers and smooth muscle cell (SMC) markers in $CD14^+$ monocytes. This study may provide a better understanding of biological functions of adiponectin and its potential clinical applications.

Materials and methods

Chemicals and reagents

Ficoll-paque plus was purchased from Amersham Biosciences (Piscataway, NJ, USA). Pre-separation filter, human CD14 microbeads and LS separation columns were purchased from Miltenyi Biotec Inc.(Auburn, CA, USA). Human fibronectin-coated culture plates, PE-conjugated mouse IgG1, PE-conjugated anti-mouse IgG1, mouse IgG1, mouse anti-human eNOS, cytoperm/cytofix and perm wash buffer were purchased from BD Biosciences (Franklin Lakes, NJ, USA). EC basal medium-2 (EBM-2) and EGM-2 Singlequot were obtained from Clonetics (Walkersville, MD, USA). Recombinant human adiponectin and PE-conjugated mouse anti-human vascular endothelial growth factor receptor-2 (VEGFR-2) were purchased from R&D Systems (Minneapolis, MN, USA). Trizol reagent was obtained from Sigma (St. Louis, MO, USA). IQ SYBR Green supermix kit was obtained from Bio-Rad (Hercules, CA, USA).

Isolation and culture of CD14+ monocytes

Peripheral blood mononuclear cells (PBMCs) from blood donors at the gulf coast regional blood center (Houston, Texas, USA) were isolated by Ficoll-Paque density gradient centrifugation. PBMCs were then allowed to run through the pre-separation filter to remove any cell cluster. Filtered PBMCs were magnetically labeled by incubating with human CD14 microbeads in 4°C for 15 min. After washing steps, cell suspension was loaded on the magnetic LS separation column. $CD14^+$ monocytes were bound to the magnetic column and $CD14^-$ cells run through the magnetic column. The magnetic column was rinsed for several times, and then $CD14^+$ monocytes were firmly flushed out into the collection tube by using the plunger. $CD14^+$ monocytes were cultured on the human fibronectin-coated 6 well plate (1×10^6 cells/well) in complete EC growth madia-2 (EGM-2) (EBM-2 supplemented with EGM-2 Singlequot and 10 % fetal bovine serum). Adiponectin (10 μg/ml) was applied upon seeding the cells. After 2 days of culture, non-adherent cells were discarded and the fresh medium with or without adiponectin was added. The cul-

Table 1 Primer Sequence for Real-Time PCR *

Gene	Gene bank No.	Forward primer	Reverse primer
eNOS	NM_000603	AGGAACCTGTGTGACCCTCA	CGAGGTGGTCCGGGTATCC
Calponin	NM_001299	GAAACAAGGTGAACGTGGGAGTG	TTCCGCCCTTCTCTTAGCTTCC
SMMHC	NM_002474	CGGTCCCTGGCAATTCTGTTTAC	CTTGGTGAACTGTGCGTGTCTG

* Primers for all tested genes were designed *via* the Beacon Designer 2.1 software (Bio-Rad Inc., Hercules, CA).

ture medium was then replaced every 2 days. The attached cells were continually cultured in the complete EGM-2 medium with or without adiponectin over the course of 10 days. Morphological changes of adherent cells were visualized with Olympus phase-contrast microscopy over culture (Olympus Optical Co. Ltd, Tokyo, Japan).

Flow cytometry analysis

The attached cells were detached with trypsin/EDTA solution and then suspended in cold staining buffer (PBS containing 2 % FBS and 0.09% sodium azide). Before incubating with the primary antibody, the cell suspension was immnunoblocked in 10% human serum in 4°C for 20 min. Before immunolabeling with eNOS antibody, the cells were permeablized with cytoperm/cytofix buffer in 4°C for 20 min. Similar numbers of cells (1×10^6) were immunostained with manufacturer-recommended concentrations of antibodies: PE-conjugated anti-human VEGFR-2 and anti-human eNOS in 4°C for 30 min. PE-conjugated mouse IgG1 and mouse IgG1 were used as isotype controls, respectively. PE-conjugated anti-mouse IgG1 was used as secondary antibodies for eNOS antigen. The cells were washed, fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), and analyzed by FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) with the CellQuest software. Vital cells were gated manually based on the forward and side light-scattering properties. Each analysis included at least 1×10^4 cell events. Data are presented as the percentage of positive cells corresponding to the gated cells and mean fluorescence intensity (MFI) in each experiment.

Real-time PCR

Total cellular RNA was isolated by Trizol reagent extraction. The genomic DNA contamination in RNA preparation was removed by using DNA-free kit (Ambion, Austin, TX), which was confirmed by the lack of detectable genomic DNA in PCR with cDNA sample yielded in reverse transcriptase (RTase)-omitted reaction (no RTase control). Total RNA (0.5 μg) was reverse-transcribed into cDNA using iScipt cDNA synthesis kit (Bio-Rad) following the manufacturer's instruction. Primers for all tested genes were designed *via* the Beacon Designer 2.1 software (Bio-Rad). The sequence of primers was shown in Table 1. The quality of individual pairs of primers was confirmed by running conventional PCR before real-time PCR to make sure there were no detectable primer dimer and non-specific products yielded. The real-time PCR reaction included the following: 250 nM primers, 50 ng cDNA, and iQ SYBR Green supermix (0.2 mM of each dNTP, 25 U/ml iTaq DNA polymerase, SYBR Green I, 10 nM fluorescein, 3 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris–HCl). Using the iCycler iQ Real-time PCR detection system (Bio-Rad), PCR cycling conditions were set as follows: 95°C for 90 sec, 40 cycles at 95°C for 20 sec, and 60°C for 1 min, and then melting curve analysis was performed on the iCycler over the range 55–95°C by monitoring iQ SYBR green fluorescence with increasing temperature (0.5°C increment changes at 10 sec intervals). Specific products were determined as clear single peaks at their melting curves. All sample measurements were performed in triplicate. Sample cycle threshold (*Ct*) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. Standard curves for all primer amplifications were generated by plotting average *Ct* values against the logarithm starting

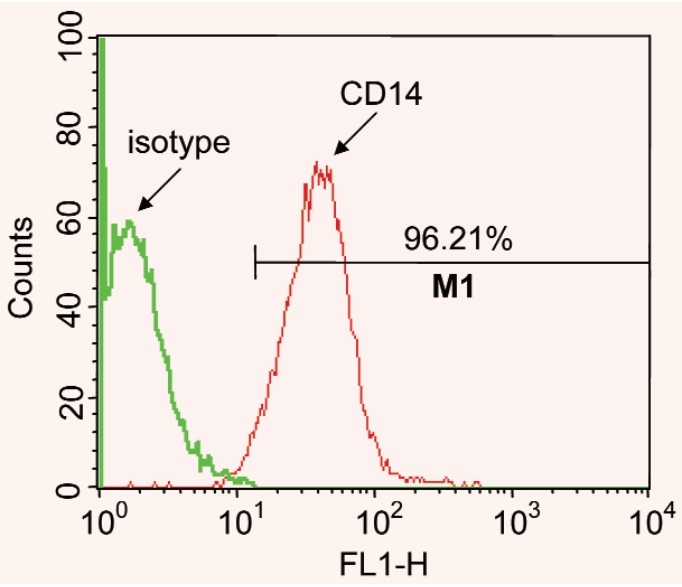

Fig. 1 Purification of CD14+ monocytes for human peripheral blood. CD14+ monocytes were isolated from human peripheral blood by specific anti-human CD14 antibody-conjugated beads. The purity of isolated CD14+ monocytes was then determined by flow cytometry analysis and was found over 95%. One of the representative histograms of flow cytometry is shown in this figure.

quantity of target template molecules (series dilution of cDNA template: 50, 10, 2, 0.4, and 0.08 ng), followed by a sum of least squares regression analysis. The correlation coefficiency and PCR efficiency of all primers were above 90%, respectively. The gene expression in each sample was calculated as $2^{(40\text{-}Ct)}$ and further normalized to β-actin expression as $[2^{(Ct\ \beta\text{-actin-}Ct\ gene)}]$.

Statistical analysis

Data from the control and treated groups were analyzed using a paired Student's t test (one tail, Minitab software, Sigma Breakthrough Technologies, Inc., San Marcos, TX). P value < 0.05 was considered statistically significant. Statistics are reported as mean ± the standard deviation (SD).

Results

Adiponectin promotes endothelial morphology formation from CD14+ monocytes.

The peripheral CD14+ monocytes were isolated from human peripheral blood by specific anti-human CD14 antibody-conjugated beads. The purity of isolated CD14+ monocytes was then determined by flow cytometry analysis and was found over 95%. One of the representative histograms of flow cytometry has been added in the revised manuscript (Fig. 1). Freshly isolated CD14+ monocytes were plated on human fibronectin-coated culture dishes and cultivated in the complete EGM-2 medium for 10 days. Morphological changes of the cells were monitored every day. Adiponectin (10 μg/ml) was added upon seeding cells. Culture medium was replaced by the fresh medium containing new adiponectin (10 μg/ml) every 2 days. After 2 days of culture, some cells attached to the bottom of the culture plate in round shape. The number of attached CD14+ monocytes in the adiponectin-treated group was much higher than that in the control group (Fig. 2A, B), suggesting adiponectin could promote the attachment of CD14+ monocytes in the EC growing medium. Upon 5 days of culture, some attached cells in the adiponectin-treated group stretched toward a spindle-shape endothelium-like morphology. Whereas, most cells in the control group remained in round shape (Fig. 2C, D). After 9 days of culture, most cells in the adiponectin-treated group showed spindle-shape endothelium-like morphology, and, particularly, some cells appeared with cobblestone-like morphology (more round and similar to mature endothelial morphology). However, only a part of the CD14+ monocytes in the control group elongated while the remaining cells were still in round shape (Fig. 2E, F).

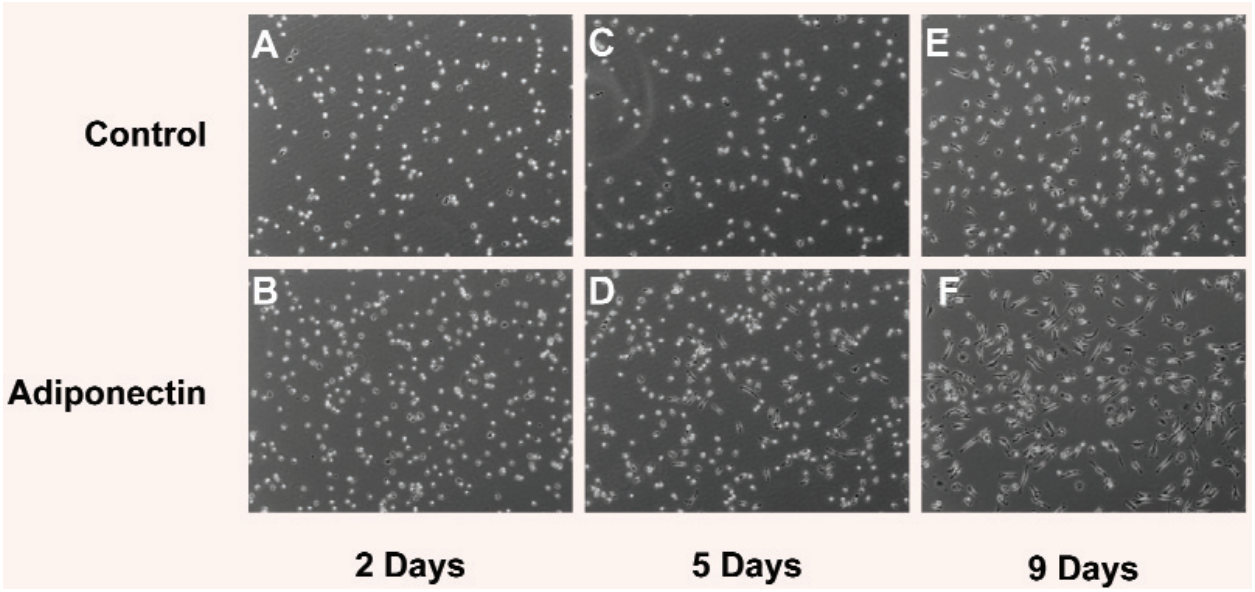

Fig. 2 Effect of adiponectin on endothelial morphology formation from CD14+ monocytes. Freshly isolated CD14+ monocytes were plated on human fibronectin-coated plates and cultured in the complete EGM-2 medium with or without adiponectin (10 μg/ml) for 10 days. Images of adiponectin-treated and control cells were acquired through camera installed on the invert phase-contrast microscopy. Representative images of day 2 (**A, B**), day 5 (**C, D**) and day 9 (**E, F**) at 200 x magnification were shown.

Adiponectin increases the expression of endothelial cell specific markers in CD14+ monocytes

In order to investigate whether CD14+ monocytes could undergo an endothelium-oriented differentiation, we performed flow cytometry to check the expression of VEGFR-2 and eNOS, two EC specific markers, in the attached CD14+ monocytes in both adiponectin-treated and control groups. We found that adiponectin stimulated the expression of VEGFR-2 and eNOS as early as 5 days of culture (Fig. 3A), and MFI of VEGFR-2 and eNOS protein was significantly increased by 49.2 % and 53.9 %, respectively, as compared to that of the control cells (Fig. 3A, $p<0.05$, n=3). In Fig. 3B, the overlay of two flow cytometry histograms representing VEGFR-2 expression of control and adiponectin-treated groups demonstrated a significant rightwards shift of VEGFR-2 expression in the adiponectin-treated group as compared to that of the control. eNOS was only detected in a very small CD14+ monocytes population (3 % ~ 5 %) as early as 5 days of culture even though the mean fluorescence intensity of eNOS was comparable to that of VEGFR-2. In addition, on the day 9 of culture, we performed real-time PCR to detect the mRNA level of eNOS in CD14+ monocytes of both adiponectin-treated and control groups. The mRNA level of eNOS in adiponectin-treated cells was significantly increased by 31.9 % as compared to that of the control cells (Fig. 4, $p<0.05$, n=3).

Adiopnectin decreases expression of SMC specific markers in CD14+ monocytes

We also checked the expression of two SMC markers, calponin and SMMHC, in the adiponectin-treated and control CD14+ monocytes using real-time PCR. At 9 days of culture, the mRNA levels of calponin and SMMHC in adiponectin-treated cells were significantly decreased by 81.1 % and 79.7 % of that of the control cells, respectively (Fig. 5, $p<0.05$, n=3).

Discussion

In this study, we reported that adiponectin at its physiological plasma concentration could promote

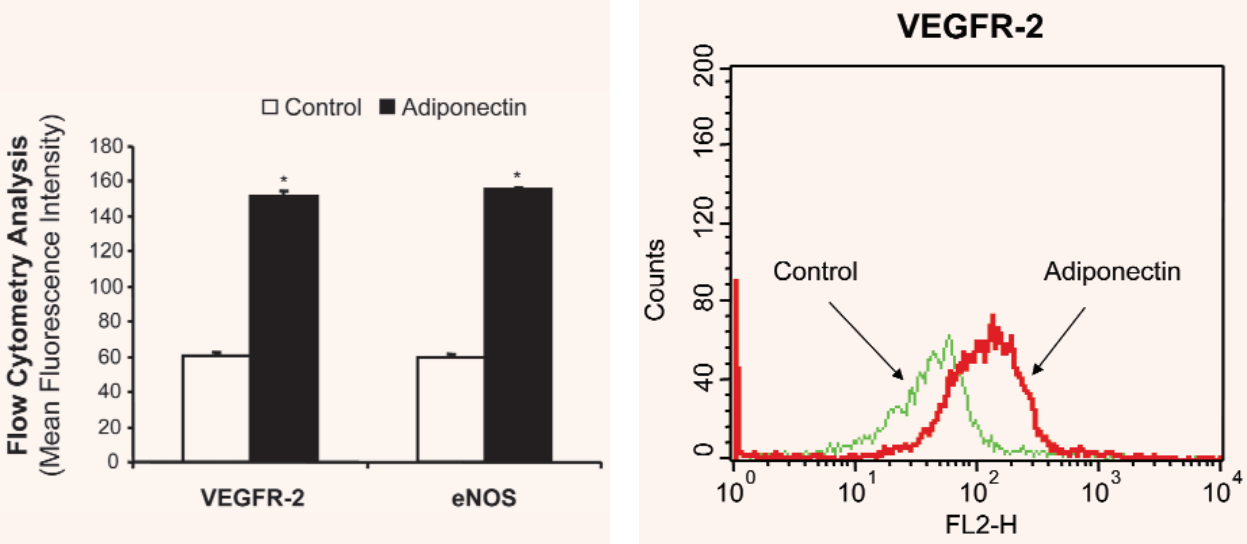

Fig. 3 Effect of adiponectin on protein expression of VEGFR-2 and eNOS in $CD14^+$ monocytes at day 5 of culture. Cells were immunolabeled with anti-VEGFR-2 antibody, anti-eNOS antibody or matched isotype controls. After fixed with 2% paraformaldehyde, cell suspension was analyzed by FACScalibur flow cytometer. Each analysis included at least $1x10^4$ cell events. Mean fluorescence intensity (MFI) for VEGFR-2 or eNOS in adiponectin-treated and control groups was acquired (**A**). The overlay of two histograms representing VEGFR-2 expression of control and adiponectin-treated groups demonstrated a significant rightward shift of VEGFR-2 expression in adiponectin-treated group as compared to that of the control group (**B**). n=3. *p<0.05

endothelial morphology formation from human peripheral blood $CD14^+$ monocytes, enhance specific EC markers VEGFR-2 and eNOS expression and decrease the mRNA levels of specific SMC markers calponin and SMMHC in $CD14^+$ monocytes. These findings may indicate a new biological function of adiponectin, which promotes endothelial cell differentiation from $CD14^+$ moncytes and favors vascular repair.

Neovascularization, a process of the formation of new blood vessels in adults, play a crucial role in restoring tissue perfusion and functions after ischemic injury due to arterial occlusive disease such as myocardial ischemia. Increasing evidence showed that circulating EPCs home to sites of ischemia and contribute greatly to neovascularization [17–21]. Adiponectin, an anti-atherogenic and anti-inflammatory adipokine,

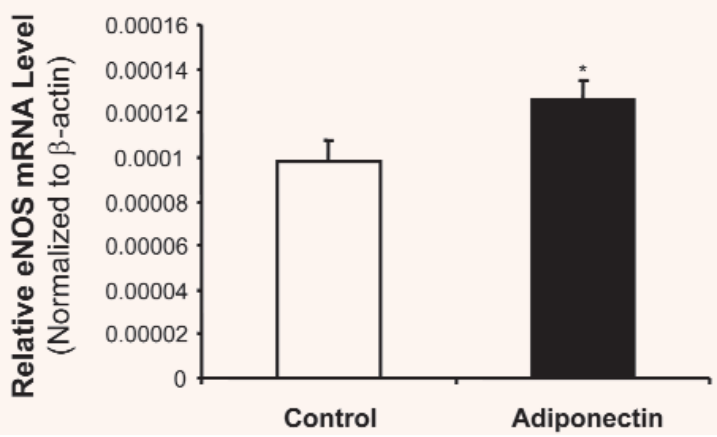

Fig. 4 Effect of adiponectin on the mRNA level of eNOS at day 9 of culture. Total RNA was extracted and then reverse-transcribed to cDNA. Fifty ng of cDNA of each sample was used in real-time PCR analysis. The eNOS expression in each sample was calculated as $2^{(40\text{-}Ct)}$ and further normalized to β-actin expression as $[2^{(Ct\ \beta\text{-actin-}Ct\ gene)}]$. eNOS, endothelial nitric oxide systhase. n=3. *p<0.05.

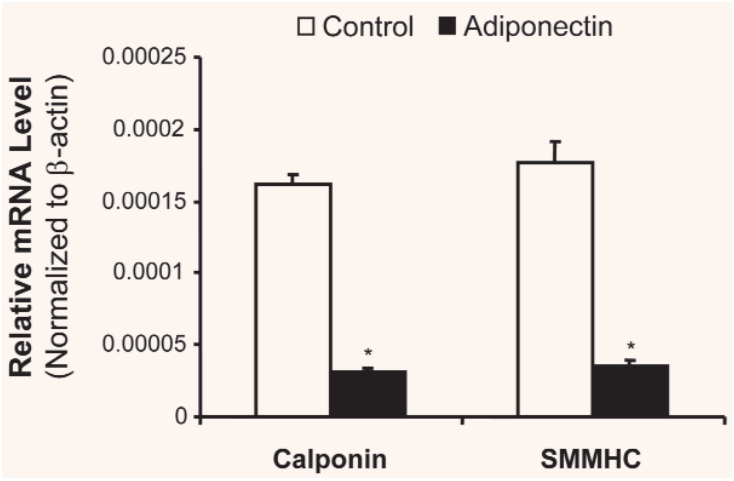

Fig. 5 Effect of adiponectin on the mRNA levels of calponin and SMMHC at day 9 of culture. cDNA preparation and analysis of real-time PCR data were previously described in Fig. 4. SMMHC, smooth muscle myosin heavy chain. n=3. *p<0.05

was recently found to be capable of stimulating angiogenesis [13–16].

Many studies showed that adiponectin promoted angiogenesis *in vivo* [13, 14]. The investigators observed that adiponectin increased endothelial cell infiltration in Matrigel plug assay, accelerated neovascularization in corneal implants and enhanced the capillary density and limb blood flow. However, there are no studies directly revealed the source of endothelial cells in the adiponectin-induced neovascularization. The mobilization and differentiation of EPCs are important in the process of adult neovascularization [17–21]. EPCs contribute as much as 25 % of endothelial cells in newly formed blood vessels [22]. $CD14^+$ monocytes are abundant in human peripheral blood and have been included in the candidates of EPCs. Since adiponectin was revealed to stimulate angiogenesis *in vivo*, we hypothesized that adiponectin may enhance the differentiation of $CD14^+$ monocytes into endothelial cells, thereby favoring neovascularization and vascular healing. In future, it is warranted to determine whether adiponectin can promote wound healing and angiogenesis *in vivo* through endothelial cell differentiation from human $CD14^+$ monocytes.

There are several sources from which EPCs could originate including embryos, bone marrow, human umbilical cord blood and PBMCs [14, 26–30]. Among those sources, PBMCs were the most clinically practical source and can be acquired from any patients without concerning about the donor and immunity-dispute response. In PBMCs, $CD14^+$ monocytes and $CD34^+$ monocytes have been revealed to possess an endothelium-differentiation capacity [26, 31, 32]. $CD14^+$ monocytes are abundant in human peripheral blood (account for 10 % ~ 20 % PBMCs), whereas $CD34^+$ monocytes are a very small population in PBMCs (only account for 0.13 % ~ 0.36 % PBMCs) [26]. Thus, we choose $CD14^+$ monocytes from PBMCs in this study to investigate whether adiponectin could promote $CD14^+$ monocytes differentiation into ECs.

Adhesion to culture plates and morphological change are the most important initial steps for EPC differentiation. We found that adiponectin promoted adhesion of $CD14^+$ monocytes to the extracellular matrix (human fibronectin), which was coated on the culture plates, and stimulated EC morphology formation from $CD14^+$ monocytes in the EC growing medium. Overall, $CD14^+$ monocytes in both groups showed a minimal proliferative capacity throughout 10 days of culture, which is consistent with the previous reports [33].

In general, the half-life of blood monocytes is about one to two days *in vivo*, whereas the life span of tissue macrophages is several months [34]. However, the *in vivo* half-life of specific subpopulations of blood monocystes is not known. Bone marrow-derived stem cells including $CD14^+$ monocytes may have a long half-life and great potential of proliferation and differentiation. *In vivo* conditions are definitely far more complicated than *in vitro* conditions. Although the half-life of circulating monocytes is estimated only one to two days, these cells will acquire a long life span as several months when they enter the tissues and become tissue macrophages. In order to investigate the effect of adiponectin on $CD14^+$ monocytes differentiation

in vitro, we treated the $CD14^+$ monocytes, which adhered to the culture plate for up to 10 days without cell death, which is consistent with many other *in vitro* investigations [26, 30].

To elucidate whether $CD14^+$ monocytes could differentiate into ECs, we studied the EC-specific marker expression in $CD14^+$ monocytes as an index of endothelium-oriented differentiation. VEGFR-2 and eNOS are two EC-specific markers and are generally selected in evaluating EPC differentiation. In this study, adiponectin significantly increased the protein expression of VEGFR-2 and eNOS. VEGFR-2 is the pivotal receptor mediating the mitogenic action of VEGF. VEGF is known to play an essential role in angiogenesis and neovascularization, and it is also known to be critical in EPC differentiation. VEGF can promote $CD14^+$ monocytes proliferation in the EGM-2 medium and the formation of ultrastructures resembling Weibel-Palade bodies in these cells [26, 35]. VEGF is applied to induce pluripotent stem cells from PBMCs to differentiate into ECs [37]. EPCs themselves from PBMCs are reported to release VEGF which may in turn enhance EPC differentiation and proliferation [33]. VEGF promotes endothelial cell maturation through interaction with its specific receptors, especially VEGFR-2. VEGF elevation has been found to be associated with rapid mobilization of a population of VEGFR-2-positive EPCs [37]. Also, VEGFR-2-positive cells derived from embryonic stem cells are found to serve as "vascular progenitor cells", and VEGF promotes these cells differentiating into ECs [38]. Evidence has shown that posterior mesodermal VEGFR-2-positive cells have the capacity to differentiate into ECs [39]. VEGFR-2-mediated signal is required for the migration and expansion of EPCs [40]. In the complete EGM-2 medium, there exists abundant VEGF. In this study, adiponectin could effectively upregulate VEGFR-2 expression in $CD14^+$ monocytes, which may thus enhance VEGF-induced $CD14^+$ monocyte differentiation into ECs through the VEGF-VEFGR-2 signaling pathway. In addition, adiponectin is recently revealed to stimulate AMP-activated protein kinase (AMPK)-PI3-AKT signaling pathway to enhance EC proliferation and migration [13, 14] and inhibit EC apoptosis [15, 16]. AMPK-PI3-AKT signaling pathway is one of the essential signaling pathways mediating VEGF-VEGFR-2 signals. VEGF promotes vascular homeostasis and angiogenesis through its ability to activate Akt signaling [41]. Akt signaling also regulates VEGF-stimulated EC differentiation and migration [41, 42]. AMPK signaling is required for VEGF-stimulated EC NO production, migration and differentiation [43]. In our case, it is required to inhibit PI3K activity for a long time (up to 9 days) in order to study the role of AMPK-PI3K-AKT signaling pathway in the adiponectin-induced CD14+ cell differentiation. However, the chemical inhibitors, *i.e.* LY294002 or wortmanin, usually work for only a short time (12–30 hrs). Thus, it is impossible for us to use these inhibitors in our study due to the related long-term observation. In future, it is possible to use other approaches such as PI3K siRNA expression vector or adenoviral vector expressing dominant-negative PI3K to specifically downregulate PI3K expression or activity to study the role of AMPK-PI3K-AKT signaling pathway in the adiponectin-induced $CD14^+$ cell differentiation.

In current study, eNOS was also upregulated in adiponectin-treated $CD14^+$ cells. Adiponectin has been known to promote phosphorylation of eNOS and thus enhance eNOS activity and NO production through activating AMPK-PI3-AKT pathway or promoting the association between HSP90 and eNOS [13, 44, 45]. Increasing evidence shows that eNOS-NO pathway is essential in promoting EPC recruitment, survival and differentiation. CRP, a key predictor as well as mediator of atherosclerosis and other coronary artery diseases, could inhibit EPC survival and differentiation through impairing eNOS-NO pathway [25]. Stromal cell-derived factor-1 α gene transfer could enhance ischemia-induced vasculogenesis and angiogenesis *in vivo* through a VEGF/eNOS related pathway [46]. Estrogen-mediated, eNOS-dependent mobilization of bone marrow-derived EPCs could contribute to reendothelialization after arterial injury [47]. In current study, elevated activity of the eNOS-NO pathway might be another molecular mechanism of adiponectin inducing EC differentiation from $CD14^+$ cells.

Progenitor cells are primitive cells and possess a multiple-lineage differentiation capacity under different conditions [31, 36]. Some investigators reported that EPCs can differentiate into EC under shear stress stimulation [48] and into SMCs under cyclic strain stimulation [49]. Hence, we investigated the expression of two SMC markers to confirm

that the potential of SMC differentiation from $CD14^+$ monocytes would be suppressed under the designated condition favoring EC differentiation in current study, we found that the mRNA levels of calponin and SMMHC in adiponectin-treated $CD14^+$ monocytes were significantly decreased on the day 9 of culture. Other studies also showed that adiponectin was capable of binding with PDGF-BB and inhibiting growth factor stimulated ERK activation in human aortic SMCs (HAoSMCs), thereby strongly suppressing HAoSMC proliferation and migration [50]. PDGF-BB is known as a key growth factor for maturation and proliferation of SMCs through the ERK signaling pathway. In the complete EGM-2 medium, there are a variety of growth factors including certain amount PDGF-BB, adiponectin might inhibit the expression of SMC markers in $CD14^+$ monocytes through specific interaction between adiponectin and PDGF-BB.

Decreased plasma adiponectin levels are observed in patients and animal models with obesity, coronary artery disease and type 2 diabetes. In these cases, SMCs usually undergo uncontrolled proliferation and migration in response to a variety of cytokines and growth factors due to endothelial cell dysfunction and the loss of intimal integrity, thereby leading to progression of atherosclerosis, restenosis and hypertension and in turn worsen tissue ischemia. It is reported that adiponectin strongly suppressed human aorta SMC proliferation and migration through direct binding with PDGF-BB [50] and inhibited the SM-α-actin positive atherosclerosis lesion formation in aortic sinus in Apo-E deficient mice [8], suggesting that adiponectin acts as a modulator for vascular remodeling and maintaining the homostasis of circulation. It is indicated in our study that adiponectin decreased the potential of $CD14^+$ monocyte differentiation into vascular SMCs. This result may inhibit the uncontrolled vascular SMC proliferation and contribute to healthy vascular healing and angiogenesis.

Plasma adiponectin levels have been found to significantly decrease in patients with type 2 diabetes, obesity or cardiovascular disease. Adiponectin levels in some patients are even decreased by 30–50% as compared to that of healthy individual. Although our data can not draw the conclusion of clinical applications of adiponection, clinical association and *in vitro* effects of adiponectin on vascular cells suggests that adiponectin might be useful to treat cardiovascular diseases. In order to confirm these applications, *in vivo* investigations are warranted.

In summary, we reported, for the first time, adiponectin could promote endothelial differentiation from human peripheral blood $CD14^+$ monocytes though endothelial morphology formation and upregulation of VEGFR-2 and eNOS, and downregulation of calponin and SMMHC. Since endothelial differentiation is a critical and complicated biological process which is involved in angiogenesis, neovascularization, and vascular healing, our discoveries provide a better understanding of biological functions of adiponectin in endothelial differentiation and regulation, and suggest potential clinical applications of adiponectin in the vascular system. Further studies including *in vivo* models and dose dependent effects of adiponectin on the vascular system are warranted.

Acknowledgements

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