Int J Clin Exp Med 2014;7(1):41-50
www.ijcem.com /ISSN:1940-5901/IJCEM1311023

Original Article

Hypoxia induced changes of SePP1 expression in rat preadipocytes and its impact on vascular fibroblasts

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Received November 29, 2013; Accepted December 23, 2013; Epub January 15, 2014; Published January 30, 2014

Abstract: Human adipose tissues secret a lot of cytokines involved in physiological and pathological activities. Inflammation around blood vessels is positively related to the severity of atherosclerosis. This study was to investigate the impact of adipokine SePP1 on vascular fibroblasts (VF) under a hypoxia condition might provide new evidence and methods for treatment of atherosclerosis. The mRNA and protein expression of IL-6, MCP-1 and SePP1 were detected in preadipocytes under normoxic (21% O_2) and hyperoxic (4% O_2) conditions, and the impact of IL-6, MCP-1 and SePP1 on VF was investigated. The preadipocytes were cultured under normoxic and hypoxic conditions. Then, the cell growth, and the mRNA and protein expression of inflammatory cytokines (IL-6, MCP-1 and SePP1) were detected. The VF were cultured in the medium collected from preadipocytes maintained under hypoxic and normoxic conditions, and the phenotypes, migration and type I collagen protein of VF were determined. Results showed that under the hypoxic condition, the proliferation of preadipocytes increased significantly ($P<0.05$), and the mRNA and protein expression of IL-6 and MCP-1 elevated markedly ($P<0.05$). However, the SePP1 expression reduced dramatically ($P<0.05$). After co-culture with VF, the VF transformed into myofibroblasts, accompanied by increased migration and elevated type I collagen expression ($P<0.05$). Thus, hypoxia may accumulate visceral fat and induce inflammatory state of preadipocytes, with reduced SePP1 expression, which might be involved in the occurrence and development of atherosclerosis.

Keywords: Preadipocyte, vascular fibroblasts, hypoxia, SePP1, monocyte chemoattractant protein-1, interleukin 6, type I collagen

Introduction

Vascular fibroblasts (VF) are major cells in the adventitia. They not only support the blood vessels but also provide nutrients to vascular media and support the sympathetic nerve endings and nourishing blood vessels. Thus, VF plays important roles in the maintenance of structural and functional stability of blood vessels [1, 2]. Under pathological conditions, the adventitial remodeling of coronary artery precedes the presence of endothelial dysfunction [3]. The adventitia is no longer a traditionally considered bystander, but together with intima and media participates in the occurrence and development of cardiovascular diseases including atherosclerosis.

Under normal conditions, adventitia is closely connected to the surrounding loose connective tissues and usually capsulated by surrounding adipose tissues. With the investigation of secretion of adipose tissues, increasing attention has been paid to the perivascular adipose tissue (PAT). SePP1 is one of cytokines synthesized and secreted by adipose tissues and can influence the differentiation of adipose tissues via regulating the oxidative stress and inflammation [4]. There is evidence showing that SePP1 has the activity of Phospholipid hydroperoxide glutathione peroxidase and can directly exert anti-oxidative effect.

Materials and methods

Animals and reagent

Healthy adult SD rats (Male; 6-8 weeks) were purchased from Shanghai Slyke Experimental Animal Co., Ltd. 0.25% trypsin-EDTA, fetal bovine serum, penicillin, streptomycin (Gibco), type I collagenase, oil-red O, insulin, dexameth-

asone (Sigma) and rat IL-6, MCP-1, SePP1 and type I collagen ELISA kits (R&D, USA) were used in the present study.

In vitro culture of preadipocytes

Male SD rats were sacrificed. The perinephric fat was separated, cut into blocks and mixed with 0.2% type I collagenase (v/v: 1:2) followed by digestion at 37°C for 1 h. Then, filtration was done, and the filtrate was harvested followed by centrifugation at 37°C for 5 min at 1500 rpm/min. The supernatant was removed, and the cells were re-suspended. The cell suspension was seeded into a flask at a density of $4\times10^4/cm^2$. Cells were maintained in 10% FBS DMEM/F12 at 37°C in an environment with 5% CO_2 for 24 h. Cells were digested with 0.25% trypsin for passaging, and then cells were seeded into an aseptic flask at a density of $4\times10^4/cm^2$. Cells were grown in 10% FBS DMEM/F12 at 37°C in an environment with 5% CO_2 and 21% O_2 or 5% CO_2 and 4% O_2. Medium was refreshed once every 2-3 days.

Identification of preadipocytes

The preadipocytes of the third generation was harvested and maintained in induction medium (insulin: 850 nmol/L, dexamethasone: 50 nmol/L and 10% FBS in DMEM/F12). Three days later, cells were maintained in DME/F12 containing 850 nmol/L insulin and 10% FBS for 7 days. Oil red O staining was done for identification of preadipocytes.

In vitro culture of VF

Healthy male SD rats were sacrificed, and the thoracic aorta was collected. The intima and media were removed, and tissue culture was performed at 37°C in an environment with 21% O_2 and 5% CO_2 for the separation of VF. Medium was refreshed once every 2-3 days, and cells of the third generation were harvested for experiments.

Cell proliferation assay

After culture under different conditions, preadipocytes were harvested by digestion and cell suspension was prepared followed by cell counting. The cell suspension was added to aseptic 96-well plates (2000 cells/cells) and 5 wells were included in each sample. Following incubation with CCK-8, absorbance was measured at 450 nm. The proliferation of VF was measured with identical procedures.

Detection of mRNA expression with RT-PCR

Primers were designed with the Primer Premier 5.0. The designed primers were compared with BLAST primers from NCBI to determine their specificity. Sequence of the internal reference was provided by the Tissue Engineering Laboratory of Ninth People's Hospital of Shanghai Jiaotong University. The primers and temperature for annealing were as follows: IL-6 CTGATTGTATGAACAGCGATG (forward), AACTCCAGAAGACCAGAGC (reverse) and 52°C; MCP-1: CTGATTGTATGAACAGCGATG (forward), AACTCCAGAAGACCAGAGC (reverse), and 59°C; SePP1: GGTGTCAGATCACATTGCTGTT (forward), GTAGCCGAGGACACGTTTTTAC (reverse), and 60°C; β-actin: GGTGGGTATGGGTCAGAA (forward), TGCATCCTGTCAGCGATG(reverse), and 59°C. Conditions for PCR were as follows: 95°C for 3 min, 32 cycles of 95°C for 45 s, 59°C for 45 s and 72°C for 60 s and a final extention at 72°C for 10 min.

Detection of protein content with ELISA

Preadipocytes and VF were cultured under normoxic and hypoxic conditions. After incubation under the serum free condition for 1 d, the medium was collected into a centrifuge tube, and centrifugation was done at 1500 r/min for 5 min. The supernatant was collected and stored at 4°C. Detection of contents of IL-6, MCP-1 and SePP1 in the supernatant of preadipocytes and content of type I collagen in the supernatant of VF was done with corresponding ELISA kit.

Immunofluorescence staining

VF was cultured in vitro under hypoxic and normoxic conditions in the medium collected from preadipocytes maintained under the hypoxic and normoxic conditions. Three groups were included: Group A: blank control group, Group B: hypoxia group and Group C: normoxia group. Cells were fixed and processed for immunofluorescence staining of α-actin. In brief, cells were fixed in 4% paraformaldehyde and the treated with 0.25% Triton. Cells were then blocked in 10% goat serum at 37°C for 30 min, and treated with primary antibody (1:100) at 37°C for 60

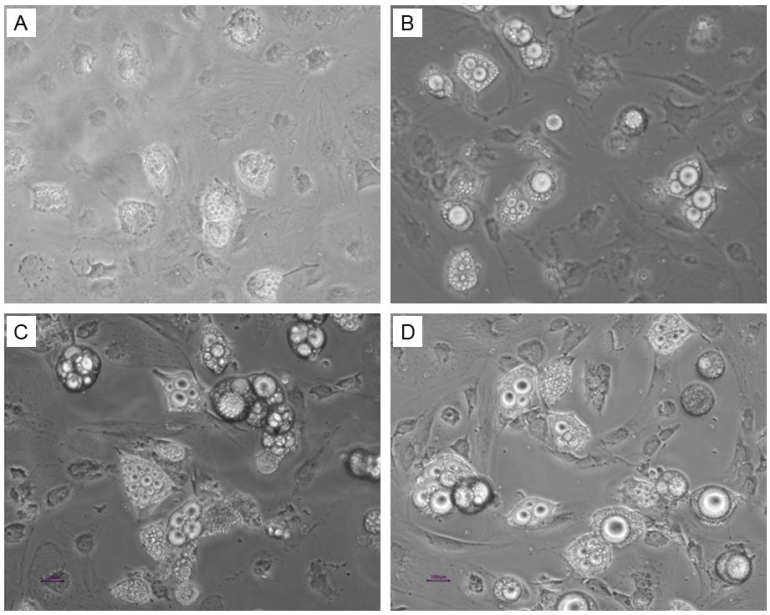

Figure 1. Morphology of perirenal preadipocytes. A: After incubation for 3 days; B: After incubation for 5 days; C: After incubation for 7 days; D: After incubation for 9 days.

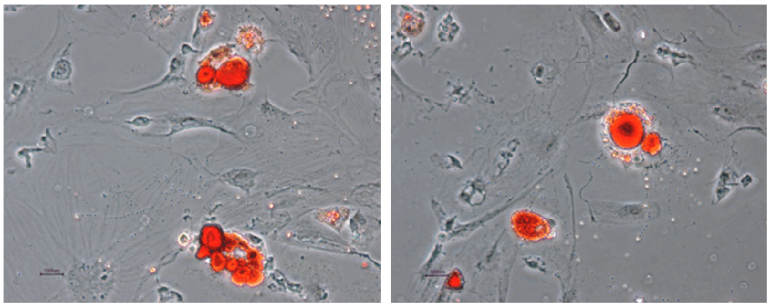

Figure 2. Oil red O staining of preadipocytes after induction.

min. In the blank control group, BSA of equal volume was added. Subsequently, cells were incubated with secondary antibody (goat anti-rabbit antibody in PBS; 1:50) at 37°C for 30 min. After nuclear staining with DAPI (1:100) for 10 s, mounting was done, and cells were observed under a fluorescence microscope.

Detection of VF migration with Transwell assay

VF was cultured *in vitro*. When the cell confluence reached about 100%, cells were starved for 24 h and then digested with trypsin and re-suspended in serum free DMEM. The cell density was adjusted to 1×10^9/L. Then, 200 μL of cell suspension was added to the upper chamber and the medium, which was collected from preadipocytes under hypoxic, and normoxic conditions or the DMEM containing 0.4% FBS was added to the lower chamber independently. After counterstaining with hematoxylin for 6 min, the membrane was observed under a microscope.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{x} \pm sd$) and one-way analysis of variance was used for comparisons among groups. LSD-t test was used for comparisons between two groups. Once heterogeneity of variance was present, Kruskal-Wallis H test was used, comparisons were done with Mann-Whitney U between two groups and multiple testing corrections was performed with Bonferroni test. Statistical analysis was performed with SPSS version 16.0, and a value of $P<0.05$ was considered statistically significant.

Results

Induced differentiation of preadipocytes

Preadipocytes were maintained in induction medium for 3 days. The cell growth reduced significantly, cells enlarged, fat granules of different sizes were found scattered in the cytoplasm. After incubation for 5 days, spindle-shaped cells became oval or round, and the fat droplets increased in the cytoplasm and aggregated into grapes-cluster like form. In several cells, large fat droplets were found. After incubation for 8-9 days, the small fat droplets merged into large droplets and pushed the nucleus to the site close to cell membrane (**Figure 1**).

Identification with oil red O staining

After incubation in induction medium, cells underwent oil red O staining and observed under light microscope. Orange fat droplets were found in the cells (**Figure 2**). This suggests

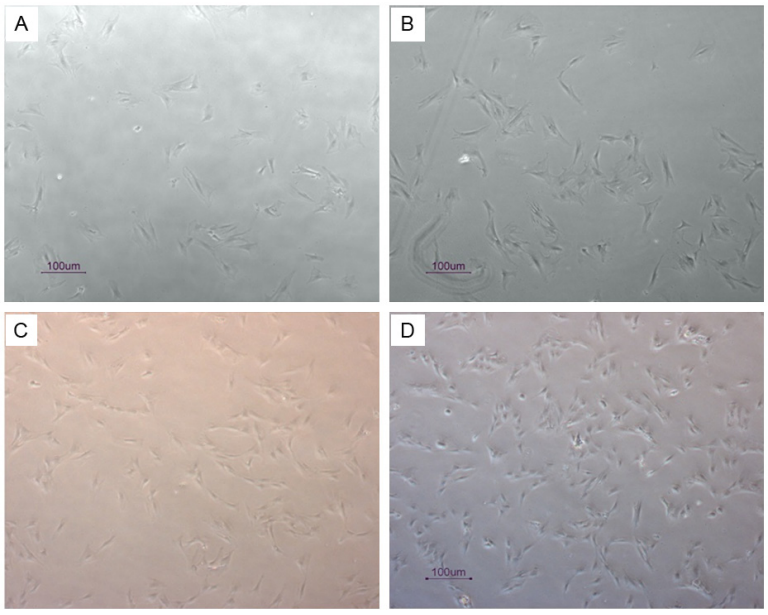

Figure 3. Preadipocytes after incubation under normoxic and hypoxic conditions. A: Cells incubated under the normoxic condition for 1 day following passaging; B: Cells incubated under the hypoxic condition for 1 day following passaging; C: Cells incubated under the normoxic condition for 3 day following passaging; D: Cells incubated under the hypoxic condition for 3 day following passaging.

Table 1. Detection of preadipocyte proliferation after incubation under hypoxic and normoxic conditions

Oxygen concentration	Absorbance at 450 nm ($\bar{x} \pm sd$)
21% O_2	0.3248±0.0212
4% O_2	0.4984±0.0235*

*P<0.01.

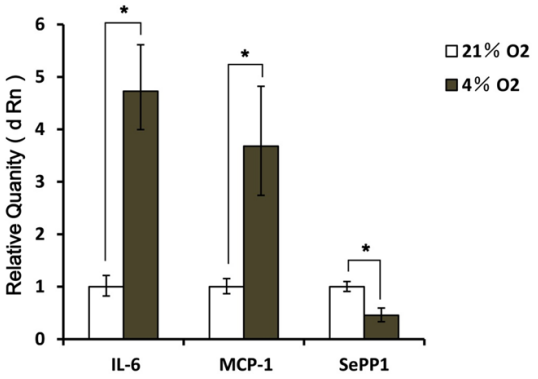

Figure 4. mRNA expression of IL-6, MCP-1 and SePP1 in preadipocytes after incubation under the hypoxic and normoxic conditions, *p<0.05 vs normoxic condition.

that preadipocytes differentiate into mature adipocytes after induction.

Detection of cell proliferation

Preadipocytes were maintained under hypoxic and normoxic conditions for 1 day, and then observed under inverted microscope. Results showed the density of preadipocytes maintained under the hypoxic condition was higher than that under the normoxic condition. After incubation for 3 days following passaging, cell density after culture under the hypoxic condition was also significantly higher than that under the normoxic condition (**Figure 3**). In addition, CCK-8 assay showed the cell proliferation after incubation under the hypoxic condition was markedly higher than that under the normoxic condition (**Table 1**; P<0.01).

Detection of mRNA expression with PCR

The mRNA expression of IL-6, MCP-1 and SePP1 was detected in preadipocytes after incubation under hypoxic and normoxic conditions. Results showed the IL-6 and MCP-1 expression after hypoxic incubation was significantly higher than that after normoxic incubation (P<0.05). However, the SePP1 expression in cells after hypoxic incubation was markedly lower than that after normoxic incubation (P<0.05) (**Figure 4**).

Detection of protein contents by ELISA

Cells were incubated under the normoxic and hypoxic conditions for 1, 2 and 3 days, and the contents of IL-6, MCP-1 and SePP1 were determined. Results showed the IL-6 and MCP-1 contents in hypoxia group were markedly higher than that in normoxia group (P<0.05 or P<0.01). On the contrary, the SePP1 content in hypoxia group was significantly lower than that in normoxia group (P<0.01). This suggests that the proinflammatory IL-6 and MCP-1 showed increased synthesis, but the SePP1 synthesis

Table 2. Contents of IL-6, MCP-1 and Sepp1 in the supernatant of preadipocytes after incubation under hypoxic and normoxic conditions (mean ± SD)

Group		IL-6 (pg/ml)	MCP-1 (pg/ml)	SePP1 (pg/ml)
Normoxia	Day 1	28.7288±1.3628	306.2532±10.7867	108.2938±5.3177
	Day 2	29.4908±3.3074	408.3358±9.8772	111.5584±3.0071
	Day 3	32.9228±2.2182	1678.0676±151.5791	873.8358±10.3480
Hypoxia	Day 1	37.0800±2.4064*	345.4944±11.7373*	60.2834±6.8083**
	Day 2	74.2348±6.2836$^{\Delta}$	508.9342±16.8541$^{\Delta}$	58.4170±5.5543$^{\Delta}$
	Day 3	1315.7296±54.8002$^{\triangledown}$	1842.7362±103.7900$^{\triangledown}$	181.4098±8.1146$^{\triangledown}$

*P<0.05 vs. Normoxia on day 1; **P<0.01 vs. Normoxia on day 1; $^{\Delta}$P<0.01 vs. Normoxia on day 2; $^{\triangledown}$P<0.01 vs. Normoxia on day 3.

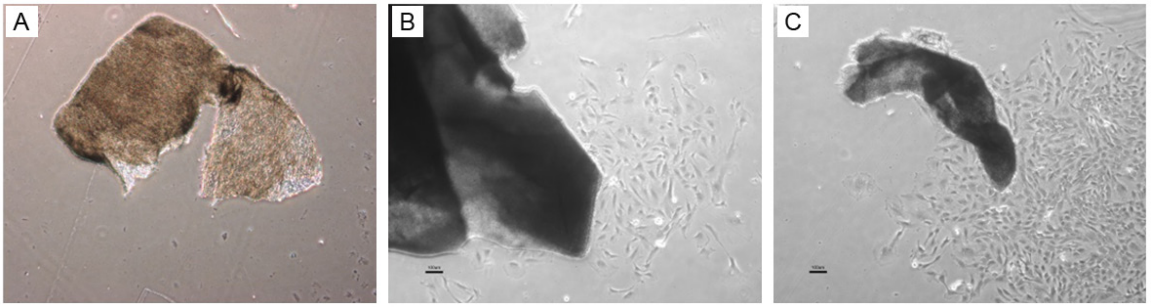

Figure 5. In vitro culture of VF (×40). A: Tissue adherent to wall; B: Fibroblasts just migrated from the tissue; C: Clusters of fibroblasts.

reduced in preadipocytes under the hypoxia condition (**Table 2**).

In vitro culture of VF

Tissue culture was performed in vitro. Six days later, cells were found to migrate from the tissues. These cells were spindle-shaped and had a radial growth and loose distribution. At 9 days after culture, cells around these tissues increased significantly and formed clusters (**Figure 5**).

Immunofluorescence staining

Under a fluorescence microscope, VF in blank control group (Group D) showed blue nuclei stained by DAPI, but green fluorescence was absent; in normoxia group (Group C), several cells showed green fluorescence; after hypoxic incubation for 3 days (Group B), a fraction of cells showed green fluorescence; after hypoxic incubation for 6 days (Group A), a lot of cells displayed green fluorescence (**Figure 6**). Statistical analysis showed the fluorescence intensity was significantly different in cells incubated under the normoxic and hypoxic conditions for 6 days (P<0.05; Group A vs. Group C). In addition, under the hypoxic condition, the fluorescence intensity on day 3 was markedly lower than that on day 6 (P<0.05). This suggests that the VF after hypoxic culture shows evident phenotype alteration (transformation into myofibroblasts) when compared with VF incubated under the normoxic condition, and the longer the incubation, the more evident the phenotype transformation was.

Detection of cell migration by Transwell assay

At 1, 3 and 5 days after culture, the membrane was observed under a light microscope, and 5 fields were randomly selected from each membrane followed by cell counting. The average was obtained, and experiment was done thrice. Results showed the VF migration after incubation under the normoxic condition was more obvious than that after incubation under the hypoxic condition (P<0.05) (**Figure 7**).

Detection of type I collage by ELISA

AF was incubated in the medium collected from preadipocytes, which were maintained under the hypoxic and normoxic conditions. After culture for 3 days, the medium was harvested for

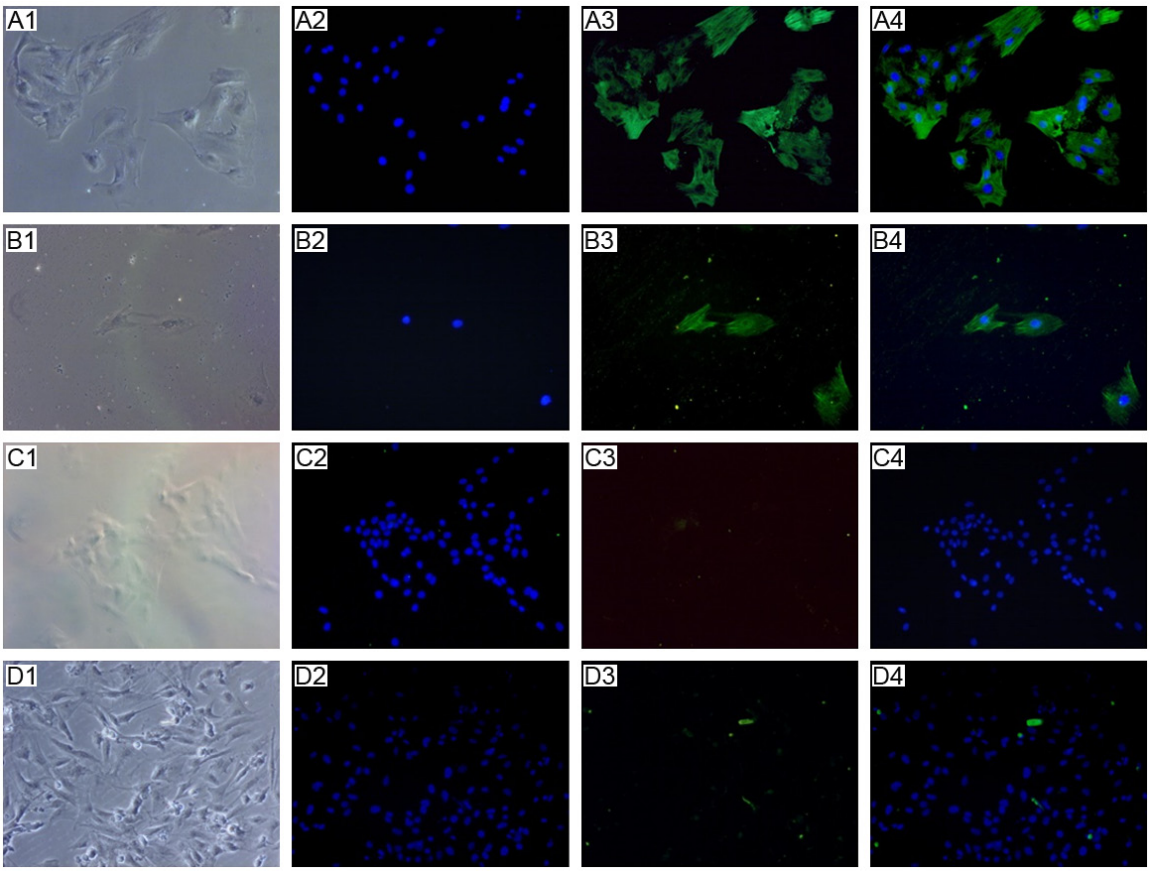

Figure 6. Immunofluorescence staining of VF after co-culture with preadipocytes (×100). A: VF maintained with preadipocytes for 6 days under the hypoxic condition; B: VF maintained with preadipocytes for 3 days under the hypoxic condition; C: VF maintained with preadipocytes for 6 days under the normoxic condition; D: VF maintained for 6 days in blank control group. 1: Cell morphology under light microscope; 2: Nuclear staining; 3: Immunofluorescent staining of a-actin; 4: Total imaging of immunofluorescent and nuclear staining.

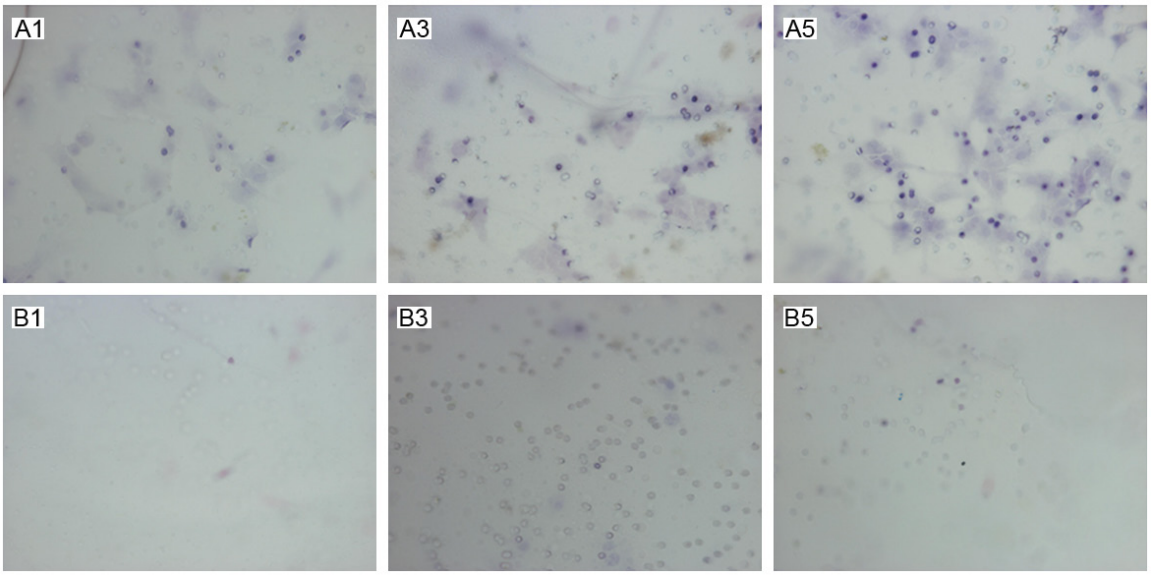

Figure 7. VF migration after incubation under normoxic and hypoxic conditions (×200). A: VE maintained in the medium collected from preadipocytes which were maintained under the hypoxic condition; B: VE maintained in the medium collected from preadipocytes which were maintained under the normoxic condition; A1 and B1: Culture for 1 day; A3 and B3: Culture for 3 days; A5 and B5: Culture for 5 days.

Table 3. Content of type I collagen in the medium of VF incubated with medium from preadipocytes (mean ± SD)

Group	Type I collagen (pg/ml)
Hypoxia	44.427±1.95
Normoxia	20.017±1.24*

*P<0.05 vs. hypoxia group.

ELISA of type I collagen. Results showed the content of type I collagen in the normoxia group was significantly higher than that in the hypoxia group (**Table 3**).

Discussion

Increasing evidence shows that adventitia plays important roles in the oxidative stress and repair of injury of blood cells. It is reported that there are inflammatory cells in the adventitia of patients with atherosclerosis. There is no infiltration of inflammatory cells in the adventitia of coronary artery of patients with atherosclerosis. Schwaz et al confirmed that the inflammation of adventitia was positively related to the severity of atherosclerosis [5]. The aggregation of T cells in the adventitia precedes the pathological changes in the arteries of patients with early atherosclerosis. Thus, in the early pathological changes of atherosclerosis patients, changes have occurred in the adventitia. That is, the fibroblasts in the adventitia participate and promote the development of atherosclerosis [6]. Studies on re-stenosis after angioplasty also revealed that the changes in the adventitia of coronary artery were the earliest in pigs with balloon induced arterial injury [7, 8]. Besides the smooth muscle cells in the media of arteries, the fibroblasts in the adventitia may also migrate into the intima to facilitate the formation of new intima. At the same time, the morphology of median and intima alters. Besides the thickening of adventitia, the components of cells and matrix also change corresponding. In addition in pigs with hypercholesterolemia and renal hypertension, the adventitia remodeling of coronary artery precedes the endothelial dysfunction [9]. These findings suggest that the adventitia is no longer a traditionally considered bystander, but together with intima and media participates in the occurrence and development of cardiovascular diseases including atherosclerosis.

In rat carotid artery balloon injury model, the fibroblasts (not smooth muscle cells) in the adventitia firstly show active proliferation, which denies the hypothesis that the newly formed intima is derived from smooth muscle cells in the adjacent media. Fibroblasts as the major cells in the adventitia may transform into myofibroblasts in the presence of stimuli (such as vascular injury, hypoxia, inflammation, and stimulation by growth factors or cytokines) [10]. These myofibroblasts may secret some matrix protein and cytokines and also show active proliferation and then migrate into the newly formed intima. In addition, these myofibroblasts may also promote the proliferation of smooth muscle cells and endothelial cells. Thus, myofibroblasts play important roles in the occurrence and development of atherosclerosis. Myofibroblasts show similarities to smooth muscle cells in the morphology and functions [11]. Myofibroblasts have contractile activity and express α-actin (not smoothelin, a marker of smooth muscle cells) [12]. In addition, myofibroblasts may also secret a lot of growth factors, cytokines and inflammation regulatory factors (such as reactive oxygen species, nitric oxide) to participate in the pathological and physiological processes including the proliferation of smooth muscle cells in the media and endothelial cells in the intima. Removal of adventitia may cause abnormal hyperplasia of intima and media. These findings indicate that the fibroblasts and myofibroblasts in the adventitia play crucial roles in the occurrence and development of atherosclerosis.

In recent years, studies have shown that adipokines or adipocytokines are involved in the regulation of multiple functions such as metabolism, cardiovascular function, inflammation related immunity and insulin sensitivity [13, 14]. The adipose tissues aggregate over age, and their distribution and functions also alter correspondingly. These changes are usually accompanied by the increase in inflammation related factors and reduction in adiponectin (a factor helpful to improve insulin resistance [15], resulting in insulin resistance and infiltration of macrophages. The abnormal aggregation, ectopic distribution and/or functional alteration of adipose tissues are related to the high risk for some common diseases such as atherosclerosis, hypertension and diabetes [16]. In the adipose tissues, 15-50% of cells are preadipocytes [17] and their differentiation determines the extent of maturation of adipose tissues. Studies have revealed that the preadi-

pocytes increase or remain unchanged over age depending on the sites, but the replication and differentiation of these preadipocytes reduced. These age and site related differences in preadipocytes are also observed in in vitro preadipocytes [18]. In addition, in the inflammation, the preadipocytes remain at an undifferentiated stage [18] and produce more pro-inflammatory cytokines, which produces a chronic inflammation status resulting in insulin resistance and metabolic syndrome [19]. Zimmermann et al found that, under specific conditions (such as hypoxia and oxidative stress), the visceral fat was different from subcutaneous fat; the expression of adiponectin and leptin was lower and the IL-6 expression was higher in the visceral fat, displaying an active fatty acid cycle and fatty acid oxidation; under this condition, the association with insulin resistance was more evident [20], and the whole body was in a transient or long-lasting chronic inflammation status [21]. In ob/ob mice and mice and rats with high fat diet-induced obesity, the SePP1 expression reduces significantly in the adipose tissues, and SePP1 has been found to regulate the oxidative stress and inflammatory response affecting the differentiation of adipose tissues [22]. In addition, SePP1 also has the phospholipid hydroperoxide glutathione peroxidase activity and may directly exert anti-oxidative effect.

The normal adventitia is closely connected to the surrounding loose connective tissues and capsulated by the surrounding adipose tissues. Thus, increasing attention has been paid to the PAT. PAT can secret a lot of factors and may involve the vascular functions and transformation of cellular phenotype via paracrine [23]. These factors may directly act on the fibroblasts in the adventitia, smooth muscle cells in the media and endothelial cells in the intima or indirectly act on these cells via the supporting blood vessels, to regulate the growth, apoptosis, function, phenotype transformation and matrix protein secretion of these cells. Thus, these factors have been found to be involved in the vascular remodeling and may recruit and activate circulating immune cells to influence the focal inflammatory environment. The internal environment of PAT is crucial for the maintenance of these factors. However, vascular injury, obesity, infection and aging may cause internal environment disorders (such as alteration of PAT function, infiltration of inflammatory cells, T cells and macrophage). This may facilitate the expression and secretion of some factors which may promote inflammation, coagulation, vascular contraction and proliferation of smooth muscle cells and fibroblasts, resulting in or deteriorating the vascular fibrosis, atherosclerosis, hypertension, intimal hyperplasia and intimal dysfunction and then leading to the occurrence and development of vascular diseases.

Visceral adipose tissues mainly locate in the abdominal cavity and capsulate some organs. They may support, stabilize, protect and regulate these organs and have important influence on the energy metabolism, hypertension, coronary artery disease and inflammation. In the present study, preadipocytes from visceral adipose tissues were cultured in vitro. After differentiation induction, oil red O staining showed orange fat droplets in these cells. This suggests that these cells were preadipocytes, which was consistent with previously reported [18].

The tissues and organs become dysfunctional and the autoregulation reduces over age, which reduce the anti-oxidative capability and the ability to scavenge free radical is also compromised. Under this condition, the body is in a relatively hypoxic state. In addition, aging and obesity may cause the internal environment being in a chronic hypoxic state [21, 24, 25]. However, the accumulation of adipose tissues may reduce the capillary density in the adipose tissues, and vascular contraction may deteriorate the reduction in blood flow. Thus, the blood supply to the adipose tissues is insufficient and the adipose tissues are in a relatively hypoxic environment [26]. In addition, obesity may also cause the increase in the oxidative stress of the body and adipose tissues [27].

Our results showed the expression of IL-6 and MCP-1 increased in the preadipocytes after hypoxic exposure, suggesting the inflammatory state in these preadipocytes under the hypoxic condition. Studies have confirmed that SePP1 has anti-oxidative activity and plays an important role in the occurrence and development of cardiovascular disease including atherosclerosis. To further investigate the influence of inflammatory state on the mRNA and protein expression of SePP1 in the preadipocytes, PCR, Western blot assay and ELISA were performed to detect the SePP1 mRNA and protein expres-

sion in these cells. Results showed the change in SePP1 expression was contrary to that in the expression of IL-6 and MCP-1 in the preadipocytes after hypoxic exposure.

There is evidence showing that SePP1 is closely related to the occurrence and development of cardiovascular diseases [18]. Whether the plasma SePP1 or SePP1 in PAT directly influences the atherosclerosis or SePP1 indirectly affects atherosclerosis via other pathways is still unclear. Our results showed VF transformed into myofibroblasts after culture in the medium from hypoxia-exposed preadipocytes, accompanied by increase in type I collagen expression and cell migration (characteristics of early atherosclerosis). However, the change in SePP1 expression is opposite. Thus, we speculate that SePP1 may influence the physiological and pathological functions of VF. However, the mechanisms underlying the influence of inflammatory state secondary to hypoxic exposure on the SePP1 expression and the mechanisms and influence of changes in SePP1 expression on the atherosclerosis are largely unclear, and more studies are required.

Disclosure of conflict of interest

None.

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