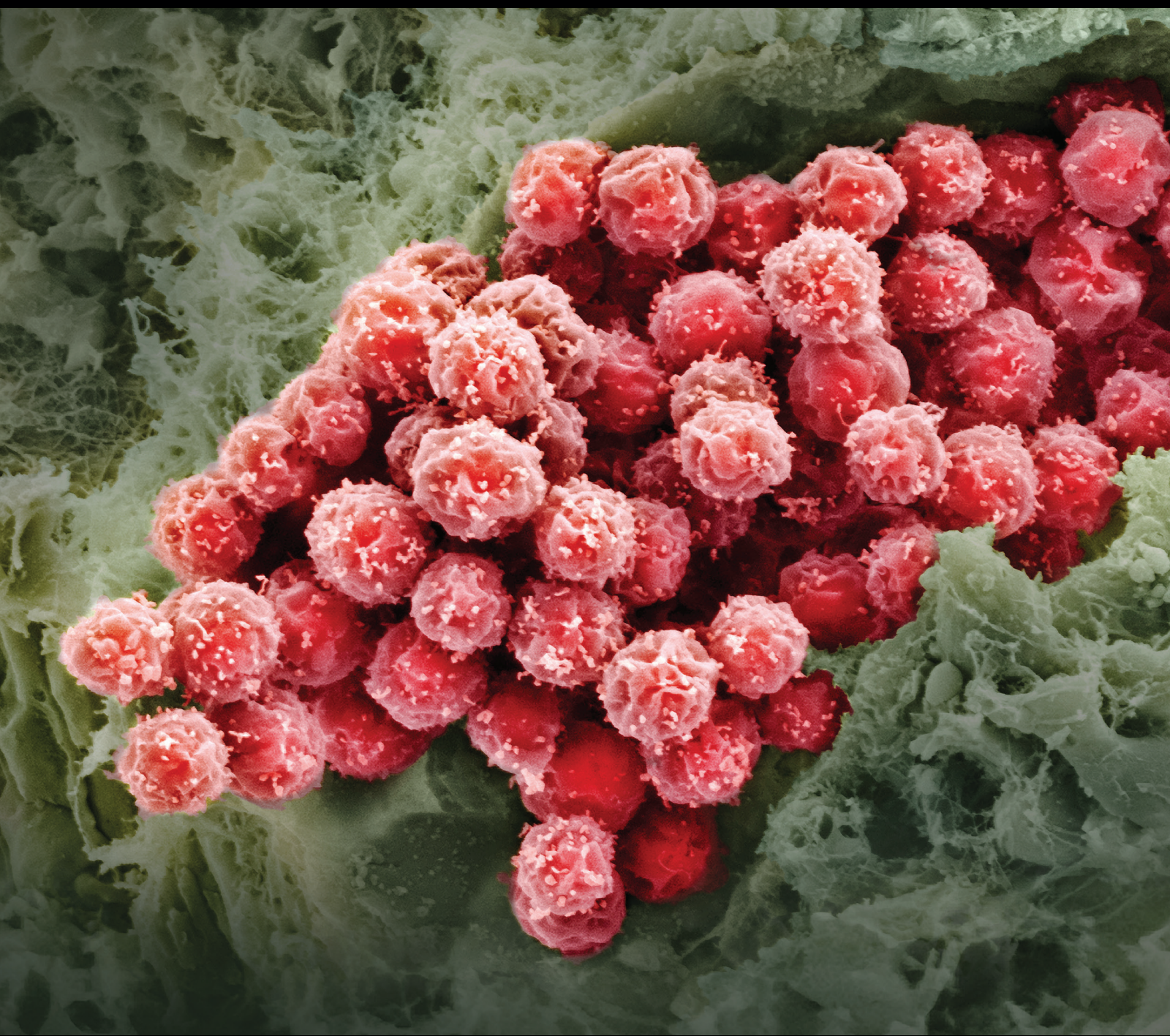
Stem Cells International

Vascular Diseases and Metabolic Disorders

Guest Editors: Ying-Mei Feng, Catherine Verfaillie, and Hong Yu



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Editorial Board

Contents

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Editorial

Vascular Diseases and Metabolic Disorders

Ying-Mei Feng,[1,2] Catherine Verfaillie,[1] and Hong Yu[3]

[1]*Stem Cell Institute, Leuven University, 3000 Leuven, Belgium*
[2]*Beijing Key Laboratory of Diabetes Prevention and Research, Lu He Hospital, Capital Medical University, Beijing 101149, China*
[3]*Zhejiang University, Hangzhou 310027, China*

Correspondence should be addressed to Ying-Mei Feng; yingmeif13@sina.com

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Vascular diseases include cardiovascular and peripheral vascular diseases. For decades, cardiovascular diseases (CVD) stay the number one mortality worldwide. In more details, coronary heart disease or stroke alone caused around 1 of every 6 deaths or 1 of every 19 deaths in the United States in 2010, respectively [1]. The total direct and indirect cost of CVD remains higher than any other diagnostic groups such as cancer [1].

In contrast to CVD, peripheral vascular diseases suffer lack of attention because most of the affected individuals are asymptomatic. The prevalence of peripheral vascular diseases is increasing which reduces the life quality and exposes the risk of infection and thrombosis. Atherosclerosis serves as the common pathogenesis of peripheral arterial disease and coronary heart disease. Therefore, both types of diseases share the same risk factors. For instance, a recent study demonstrated reduced number of endothelial progenitor cells in patients with CVD [2] and PAD [3].

Patients with vascular diseases are always featured as raised blood pressure, obesity, diabetes, and dyslipidemia, all of which constitute metabolic syndrome. From 2003-2004 to 2011-2012, the prevalence of the metabolic syndrome increased from 32.9% to 34.7% [4]. When compared to healthy controls, cardiovascular mortality was 1.6-fold higher in the subjects who had metabolic syndrome [5]. Up to date, *in vitro* and animal studies have consistently illustrated that metabolic disorders disrupt endothelium integrity, promote inflammation and thrombosis, and thus accelerate the progression of vascular diseases [6–8].

Physically, damaged endothelial cells and cardiomyocytes could be replaced by proliferation of neighboring resident cells or stem/progenitor-mediated repair. However, in the occurrence of vascular diseases and metabolic disorders, the balance between cell damage and repair is twisted. Because of its fundamental potential in self-renewal and multilineage differentiation capacity, stem cell-related therapy has developed and reformed the manner of remodeling human degenerative diseases, which could be applied for diagnosis, drug screening, and the likelihood for therapy. Among all types of stem cells, mesenchymal stem cells MSCs are one of the most promising ones for translational application. A number of preclinical studies have employed MSC for the treatment of cardiomyopathy, vascular stenosis, and corneal disease [9].

In the special issue, studies from clinical and basic research were selected that presented the current status of vascular diseases and metabolic disorders. Clinical results brought updated findings on Acute Coronary Syndrome as well as peripheral artery disease, aortic aneurysms, and diabetic microvascular complications. We were informed about the effect of stem cell therapy in the treatment of vascular diseases. The report of "type 2 diabetes mellitus susceptible to pulmonary tuberculosis" enriched our knowledge on diabetes. From the basic research aspect, we got to know more about the physiology of endothelial cells and brown adipocytes.

Ying-Mei Feng
Catherine Verfaillie
Hong Yu

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Review Article

Growth Hormone-Releasing Hormone and Its Analogues: Significance for MSCs-Mediated Angiogenesis

Xiangyang Xia,[1] Quanwei Tao,[2] Qunchao Ma,[3,4] Huiqiang Chen,[5] Jian'an Wang,[3,4] and Hong Yu[3,4]

[1]*Department of Ultrasound, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China*
[2]*Hangzhou Leading Pharmatech Co., Ltd., Hangzhou 311100, China*
[3]*Department of Cardiology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China*
[4]*Cardiovascular Key Laboratory of Zhejiang Province, Hangzhou 310009, China*
[5]*Department of Cardiology, The Second Hospital of Shandong University, Jinan, Shandong 250033, China*

Correspondence should be addressed to Hong Yu; yuvascular@zju.edu.cn

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Mesenchymal stromal cells (MSCs) are promising candidates for regenerative medicine because of their multipotency, immune-privilege, and paracrine properties including the potential to promote angiogenesis. Accumulating evidence suggests that the inherent properties of cytoprotection and tissue repair by native MSCs can be enhanced by various preconditioning stimuli implemented prior to cell transplantation. Growth hormone-releasing hormone (GHRH), a stimulator in extrahypothalamus systems including tumors, has attracted great attentions in recent years because GHRH and its agonists could promote angiogenesis in various tissues. GHRH and its agonists are proangiogenic in responsive tissues including tumors, and GHRH antagonists have been tested as antitumor agents through their ability to suppress angiogenesis and cell growth. GHRH-R is expressed by MSCs and evolving work from our laboratory indicates that treatment of MSCs with GHRH agonists prior to cell transplantation markedly enhanced the angiogenic potential and tissue reparative properties of MSCs through a STAT3 signaling pathway. In this review we summarized the possible effects of GHRH analogues on cell growth and development, as well as on the proangiogenic properties of MSCs. We also discussed the relationship between GHRH analogues and MSC-mediated angiogenesis. The analyses provide new insights into molecular pathways of MSCs-based therapies and their augmentation by GHRH analogues.

1. Introduction

Growth hormone (GH), secreted by the somatotropes in the anterior part of pituitary gland, is the predominant hormone that regulates linear growth. Its production and secretion are controlled by growth hormone-releasing hormone (GHRH), along with the somatostatin, GH itself, and downstream factors such as insulin growth factor 1 (IGF-1). GHRH and its receptors are expressed not only in the hypothalamus and pituitary but also in peripheral tissues. Thus, in addition to modulating GH release, GHRH indirectly regulates the proliferation of cells in multiple other tissues including tumor cells through a GHRH/GH/IGF-1 axis. GHRH can also directly regulate cell growth through paracrine/endocrine mechanisms by binding to the GHRH receptor on target cells. Because of this, synthetic agonists and antagonists of GHRH have attracted wide attention in recent years as global regulators of cell growth with therapeutic potential including tissue regeneration and tumor suppression, respectively. GHRH has been shown to stimulate angiogenesis in human neuroendocrine tumors by promoting VEGF secretion [1]. Agonists of GHRH applied to the post infarct myocardium improved cardiac remodeling and helped resolve ischemia [2]. GHRH antagonists have been widely used to inhibit angiogenesis and proliferation of tumor cells in prostate cancer [3], endometrial cancer [4], non-small cell lung cancer [5], and ovarian cancer [6].

Mesenchymal stromal cells (MSCs), produced in the bone marrow as well as peripheral tissues, are recognized by their plastic adherence, expression of a panel of specific cell

surface markers, and multipotent differentiation potential. In part because of their multipotency and immune-privilege properties, MSCs have been widely used to promote tissue regeneration including reconstruction of blood vessels [7, 8], cardiac repair [9], and angiogenesis [10–12]. However, the full regenerative potential of MSCs for clinical application is limited by poor posttransplantation engraftment and survival of native MSCs in the adverse microenvironment of a myocardial infarct of other ischemic circumstance [13]. Various interventions have been used with some success to enhance MSC survival including genetic modification [14], hypoxia preconditioning [15, 16], and pretreatment with chemical agents such as erythropoietin and unsaturated fatty acids [17, 18]. Work from our laboratory and others confirms that GHRH and its analogues can enhance angiogenesis in the infarcted heart and markedly enhance the regenerative properties of MSCs [19, 20]. Other laboratories have also clearly shown the converse that GHRH antagonists powerfully inhibit angiogenesis and growth of lung cancer cells [21], prostate cancer cells [22], glioblastomas cells [23], and breast cancer cells [24]. Therefore, we speculate that GHRH is a natural modulator of MSC activity, and agonists or analogues of GHRH may be the key to optimizing the regenerative properties of these cells for cardiovascular indications.

Here, we summarize current knowledge on the effects of GHRH analogues on normal and malignant cells and the potential application of GHRH analogues to optimize the proangiogenic and reparative properties of MSCs.

2. GHRH and Its Analogues

2.1. The GHRH/GH/IGF-1 Axis. The GHRH/GH/IGF-1 axis is a fundamental endocrine regulatory pathway that contributes to physical and metabolic homeostasis [26]. GHRH is synthesized and stored in the hypothalamus and transported to the pituitary gland where it activates signaling by binding to a specific receptor (GHRH-R) on the pituitary. GH is stimulated by GHRH and secreted by somatotropes in the anterior part of pituitary. Circulating GH exerts its influence by directly binding to a range of cell types with GH receptors or by indirect interaction with IGF-1 [25]. IGF-1 is produced mostly by liver and muscle and regulates cell proliferation, differentiation, and maturation in multiple tissues such as bone, cartilage, skeletal muscle, adipocyte, and cardiomyocyte. By crosstalk with the IGF-1 signaling pathway, GHRH-GH contributes to fundamental physiology, metabolism, and organismal growth including epidermis, connective tissue and bone, wound healing, and blood homeostasis including glucose and lipid control [27]. Circulating GH levels are regulated through long-loop feedback and short-loop feedback mechanisms of GHRH/GH/IGF-1 axis. Because GHRH communicates both through the GHRH/GH/IGF-1 axis and by direct binding to GHRH-R on periphery cells, there is a huge therapeutic potential for its analogues both agonist and antagonist to treat disease that may be associated with any imbalance of GHRH/GH secretion [28].

2.2. GHRH Agonists. GHRH agonists are analogues of native human GHRH that have chemically modified amino acid sequences. They were initially synthesized as substitutes to treat growth hormone deficiency (GHD) [29, 30]. Since the agonists exhibit higher activity and better stability compared with the natural GHRH, they are even more suitable for clinical application [43].

In addition to their well-documented ability to stimulate the GH secretion, GHRH agonists affect peripheral tissues by direct receptor binding and stimulating cell proliferation. Multiple cell types may be affected; for example, Dioufa et al. reported that the GHRH agonist JI-38 enhanced wound healing by activating fibroblasts via a fibroblast splice variant of the native GHRH receptor [31, 32]. The GHRH agonist MR-403 was shown to have a cytoprotective effect on rat islet cocultured with adrenal cells [44]. Recently a series of new selective and more potent GHRH agonistic analogues have been produced and are being developed for clinical application. These include MR-356, MR-361, and MR-367 [45].

For cardiovascular indications, GHRH-R was recently found to be expressed by rat cardiomyocytes and administration of exogenous GHRH blocked apoptosis and reduced the cardiac scar size after myocardial infarction. The effects correlated with GHRH-R-mediated activation of reperfusion injury salvage kinase (RISK) and other survival kinase pathways including extracellular regulated protein kinases (ERK) 1/2, phosphatidylinositol 3-kinase (PI3K)/Akt and adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), glycogen synthase kinase-3β, and the signal transducer and activator of transcription-3 (STAT3) signaling pathways [33, 34]. In an extension of this, subcutaneous administration of the potent GHRH agonist MR-409 was recently shown to exert powerful cardioprotection in a swine AMI model [35]. Application of MR-409 did not affect the heart weight/body weight index and was without any detectable adverse effects supporting safety in a large animal model. The authors concluded that systemic GHRH agonists protect the heart and preserve cardiac function during and after infarction by activating GHRH receptors on cardiomyocytes. Other studies confirm the cardioprotective, reparative functions of GHRH agonist administration and have demonstrated significant downregulation of inflammatory cytokines including IL-2, IL-6, and IL-10 in response to such agonists in vivo [36]. GHRH agonist JI-38 was shown to increase SDF-1 expression and stem cells homing in a rat model of AMI, thereby promoting cardiac repair and angiogenesis [2]. Therefore, GHRH and its agonists may play important roles in the integrity and resilience of peripheral tissues, including the heart and coronary vasculature.

2.3. Antitumor Effects of GHRH Antagonists. GHRH and GHRH-R are expressed in diverse tumor cells including human breast, endometrial, and ovarian cancers. Such locally generated GHRH circuits in tumor cells trigger bioactive and immune responses that directly impact tumor cell proliferation and expansion. Plasma GHRH levels under normal (nontumorigenic) conditions are low to undetectable because of rapid clearance and there is sparse evidence that naturally circulating GHRH contributes to tumorigenesis [46]. However, GHRH antagonists may be able to suppress the

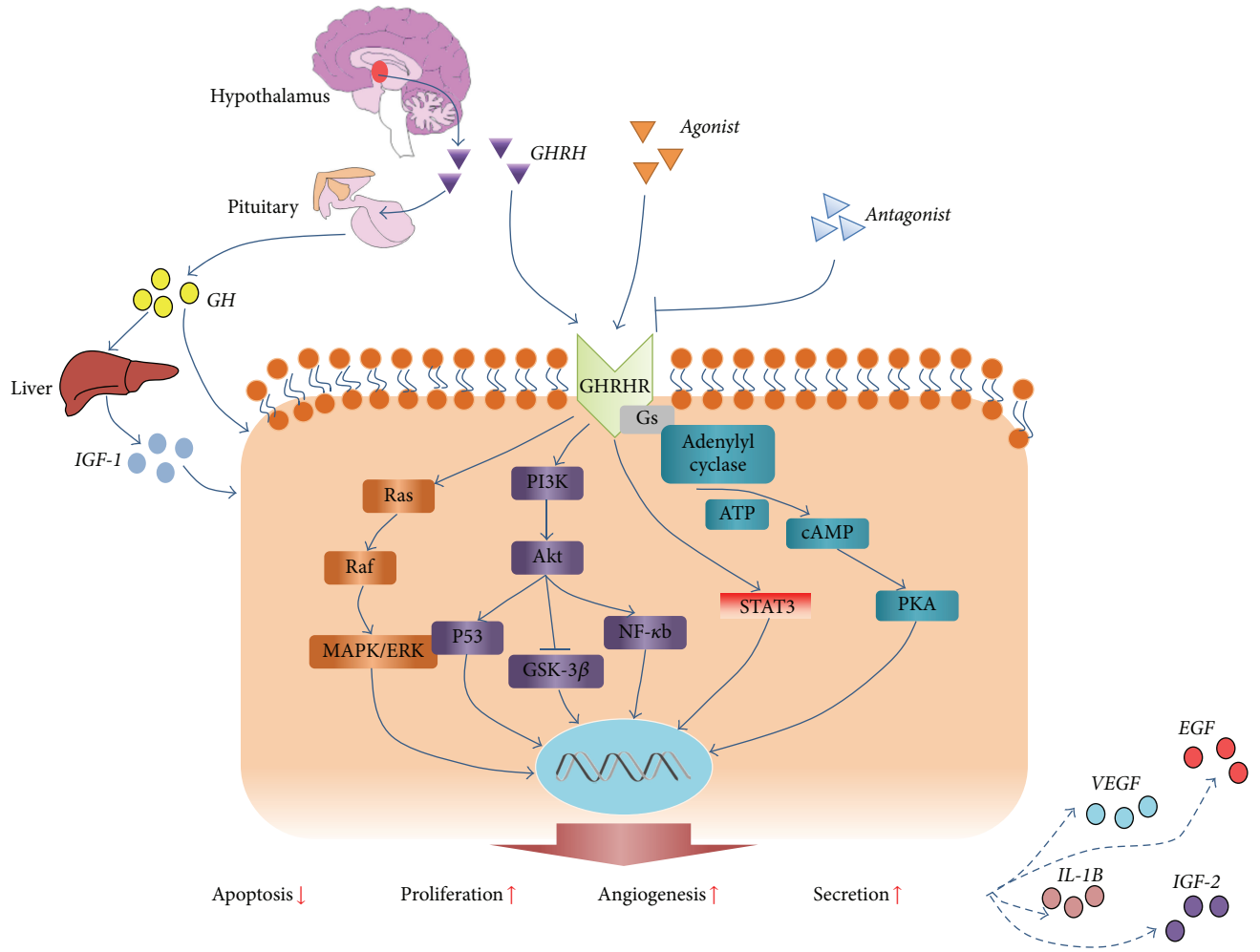

Figure 1: Cellular effects of GHRH analogues. GHRH is secreted by the hypothalamus and binds to GHRH-Rs on the pituitary to stimulate secretion of GH and downstream activity of IGF-1. GHRH and its agonists can bind directly to GHRH-Rs on multiple cell types of endocrine and nonendocrine origin. Signaling pathways that are activated by GHRH and its agonists include AC/cAMP/PKA, Ras/Raf/ERK, PI3K/Akt, and STAT3. Mediation through these signaling pathways leads to enhanced cell survival, proliferation, and secretion of cytokines. GHRH antagonists inhibit these pathways by competitively binding to the GHRH-R.

tumor progression by counteracting the localized circuits of GHRH/GHRH-R that are active in the tumor microenvironment. GHRH antagonists act as competitors of GHRH for binding to GHRH-R thereby blocking GHRH-R activation [47]. This provides us a novel approach to treat cancer with GHRH antagonists. For the past 25 years the antitumor properties of GHRH antagonists have been studied on cancer cell lines from breast, prostate, pancreas, colon, lung, ovarian, brain, and lymphocyte [23, 24, 37–42]. GHRH was shown to cause MAPK activation in MDA-MB-231 breast cancer cells via phosphorylation of Ras and Raf, and the GHRH antagonists MZ-J-7-138 and JV-1-92 were shown to block this pathway and suppress lung carcinoma growth in a manner that correlated with Ras inhibition [40, 48]. It was further demonstrated that the GHRH antagonist JMR-132 inhibited prostate cancer cell growth by suppressing Akt and ERK pathways [49]. All these studies confirm the complexity of growth regulation and signaling pathways in tumor cells that expose multiple potential targets for GHRH and its antagonists.

Angiogenesis is a central activity that controls the growth, expansion, and metastasis of tumors [50], which has been used as a primary target for the antitumor actions of GHRH antagonists. The GHRH antagonist MZ-J-7-114 was shown to block the activities of VEGF and downregulate the expression of epidermal growth factor (EGF) and VEGF receptors, thereby effectively abolishing angiogenesis and tumor growth [5]. The effects of GHRH analogues and their discriminative roles on normal versus tumor cells are summarized in Figure 1 and Table 1.

2.4. Interaction between GHRH and Other Hormones. GHRH regulation of GH secretion through GHRH/GH/IGF-1 is well established. GH secretion is also regulated indirectly by GHRH interactions with other hormones. Ghrelin is a 28-amino acid peptide produced by cells in the gastrointestinal tract that regulates GH release in a dose dependent manner

Table 1: Effects of GHRH and its analogues.

	GHRH	GHRH agonists	GHRH antagonists	Differences between GHRH and analogues
Promote GH secretion	++ [25–28]	++ [29, 30]	—	Quantitative
Cell proliferation	/	+ [31, 32]	—	Qualitative and quantitative
Cardiac protection (reduce infarct size, ameliorate cell apoptosis, and restore heart function)	/	+ [33–36]	—	Qualitative and quantitative
Antitumor effect (suppress tumor cell proliferation and angiogenesis)	—	—	+++ [23, 24, 37–42]	Qualitative and quantitative

Note: +, ++, and +++ represent positive effect: + for mild effect, ++ for moderate, and +++ for significant and strong effect; — represents suppressive effect; / represents no effect.

[51]. Ghrelin is a more potent stimulator of GH release than GHRH and the combined effect of ghrelin and GHRH on GH release is additive. GHRH and ghrelin bind independently to GHS and GHRH receptors with corresponding effects downstream [52]. Ghrelin activates the hypothalamic-pituitary-adrenal axes to regulate sleep. GHRH stimulates slow-wave sleep while corticotrophin-releasing hormone (CRH) antagonizes these pathways and stimulates wakefulness. CRH can strengthen the ghrelin-induced cortisol secretion but has no direct effect on GH release, while GHRH opposes CRH [53]. Interactions between GHRH, ghrelin, and sex steroids are synergistic and short-term beta estradiol application in postmenopausal women enhances ghrelin efficiency in the presence of GHRH [54].

3. MSCs Promote Angiogenesis

MSCs are multipotent stem cells that can differentiate into multiple cell lineages including osteoblasts, chondrocytes, adipocytes, myoblasts, fibroblasts, and stromal cells. MSCs can also be stimulated to express markers of cardiomyocytes, hepatocytes, and endothelial cells [55]. Based on their broad regenerative and immune-privileged properties, MSCs have been widely tested for use in regenerative medicine, in particular myocardial infarction and the related promotion of angiogenesis and vasculogenesis [56–64]. MSCs promote angiogenesis by the following actions: (1) secretion of trophic factors such as VEGF-A and chemoattractive cytokines [65]; (2) organization of pericytes and endothelial support cells during neovascularization [66, 67]; (3) stimulation of endogenous endothelial regenerative progenitor cells (EPCs) during ischemic injury [68]; (4) immune regulation of the microenvironment to enhance cell survival and angiogenesis [69, 70].

3.1. Paracrine Effects of MSCs. The proangiogenic stimuli of MSCs are widely believed to be mediated by paracrine pathways particularly through the actions of VEGF-A, βFGF, and SDF-1α [65, 71, 72]. Cysteine-rich protein 61 (Cyr61 or aka CCN1) is a novel proangiogenic factor that belongs to the CCN family. Cyr61 is secreted by MSCs and contributes importantly to the paracrine proangiogenic effect especially in damaged tissues [10]. Capillary growth requires degradation of the surrounding extracellular matrix (ECM) to allow endothelial cell sprouting [66]. Matrix metalloproteinases including MMP2, MMP9, and MMP14 are secreted by MSCs and target the ECM through distinct proteolytic actions thereby modulating the microenvironment and promoting appropriate interactions between MSCs and endothelial cells [66]. GATA-4 is a zinc finger transcription family that plays a key role in regulating the proangiogenic paracrine actions of MSCs. Overexpression of GATA-4 in MSCs enhances the angiogenic actions of MSCs by augmenting the secretion of multiple proangiogenic factors including VEGF-A, IGF-1, and vWF [73]. MSCs stimulate upregulation of VE-cadherin and recruitment of β-catenin to endothelial cells, which are vital for the integrity of endothelial barriers and junctions [74]. MSCs also secrete a rich mixture of cytokines and immune-modulating factors that enhance angiogenesis directly and indirectly.

3.2. MSC Function as Pericytes and Vascular Cells. MSCs can function as perivascular cells or pericytes that accumulate around the vessels in the MSC niche [8]. These cells provide structural support and may constitute a reservoir of undifferentiated precursor cells for tissue regeneration [75]. Pericytes and MSCs share common cell surface markers and are both multipotent [76]. Pericytes may be viewed as vascular MSCs that can migrate under appropriate stimulation from the MSC vascular niche to the vascular tube where they regulate the neovascularization by secreting bioactive cytokines such as VEGF-A [77]. The high migratory capability of MSCs allows them to be recruited to multiple targets in vivo, including damaged tissues and tumors such as glioma where they can integrate as pericytes. As such MSCs may be used as selective antitumor drug transfer vehicles [78].

It has also been shown that the pericytes or vascular MSCs present in the aortic artery may modulate restenosis after arterial injury. Engrafted MSCs with endothelial-like phenotypes were shown to express endothelial nitric oxide synthase (eNOS) and may retard the processes of restenosis [79]. In contrast to this, other research suggests that MSCs preferentially migrate to the medial zone of blood vessels and differentiate into vascular smooth muscle cells [80]. Therefore, the direction of MSC differentiation may be related to the tissue specific microenvironment. When cultured

under endothelial differentiation conditions, MSCs express endothelial traits and markers possibly through the upregulation of forkhead box protein C2 (FOXC2) and its downstream target alpha v beta 3 integrin/CD6 [81]. VEGF-A stimulation of cultured MSCs also promotes endothelial-like differentiation by activating the Rho/Rho-associated coiled-coil containing protein kinase (ROCK) signaling pathway and myocardin-related transcription factor-A (MRTF-A) [82]. Physical stimuli such as modeled microgravity with or without VEGF can direct MSCs to express endothelial markers including Flk-1 and vWF [83, 84]. Taken together these results confirm the multipotency of MSCs and support major roles in vascular regeneration including possible direct contributions to endothelial cell recruitment and sprout formation.

3.3. Stimulation of Endogenous Regenerative Programs. EPCs may contribute to the structure, organization, and architecture of regenerating blood vessels. In addition to their potential to generate mature endothelial cells, EPCs also mediate paracrine actions by secreting angiogenic cytokines especially at the early stages of vessel formation [85]. EPCs and MSCs cross-communicate at multiple levels and when cocultured both types of cells demonstrated enhanced proliferation and proangiogenic properties [86]. MSC-EPC intercellular signaling involves both paracrine effects and direct cell-to-cell communication [87, 88]. Simultaneous tissue transplantation of MSCs with EPCs promotes angiogenesis in a synergistic manner and conditioned medium from cocultures of EPCs and MSCs generate complementary sets of angiogenic cytokines and more efficiently promote angiogenesis of cultured endothelial cells under both normoxic and hypoxic culture conditions [89]. The results support synergism between EPCs and MSCs at multiple levels in the developing, regenerating vasculature, particularly involving secreted cytokines that promote cell recruitment, proliferation, and differentiation [90].

3.4. Immune Regulation. MSCs modulate immunoreactions by interacting with immune cells. MSCs inhibit the proliferation and maturation of B-cells and NK-cells and suppress the proliferation of $CD4^+$ and $CD8^+$ T-cells while wielding protective effect on other cells such as neutrophils [91]. MSCs can also inhibit lymphocyte proliferation as well as B-cells differentiation [55]. However, the precise mechanisms of MSCs-related immune regulation properties are not fully understood. A number of distinct pathways have been described. Firstly, MSCs can modulate immune cells by secreting related chemokines and cytokines. Activation of Toll-like receptors 3 and 4 on MSCs increases the production of IL-6, IL-8, and chemokine (C-X-C motif) ligand 10 (CXCL10) to suppress the proliferation of T-cells through Notch signaling [92]. Secondly, MSCs attract proinflammatory M1 macrophages through secreted cytokines CXCL2, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and growth-regulated protein β [69, 93]. Thirdly, MSCs can recruit anti-inflammatory M2 macrophage by accumulated production and secretion of PEG2 under mediation by γ-IFN to balance the inflammatory responses [94]. Furthermore, MSCs can directly contact to endothelial cells by the functional adherens junctions mediated by VE-cadherins and β-catenin to maintain intact endothelial barriers. Endothelial barriers can prevent uncontrolled inflammation that would be caused by increased vascular permeability [74]. Immune regulation by MSCs helps support an appropriate microenvironment that is conducive to angiogenesis and the maturation of new blood vessels.

3.5. Preconditioning to Enhance the Viability of MSCs. Despite their unique properties, clinical applications of MSCs for tissue protection and regeneration are limited by poor survival and limited engraftment. Multiple preconditioning stimuli have been tested to improve posttransplantation survival and function. These include gene modification, physical and chemical preconditioning, and the pretransplantation culture microenvironment.

Physical preconditioning includes hypoxia preconditioning [95, 96], which can attenuate apoptosis caused by hypoxia/reoxygenation. Our group has shown that the superior performance of MSCs conferred by hypoxia precondition is mediated at least in part through a leptin-mediated mechanism [15]. Hydrogen gas and oxidative stress pretreatments were also successful preconditioning stimuli that improved survival of pretransplantation of MSCs [97, 98].

Paracrine stimulation by multiple growth factors, cytokines, and chemicals can confer protection and increased survival. The repertoire includes growth factors such as TGF-β [99], TGF-α [99], erythropoietin (EPO) [17], epidermal growth factor [100], parathyroid hormone [101], and K-channel activator diazoxide [102, 103]. Such preconditioning can be implemented by pretreatment with recombinant proteins or by gene modification. For example, MSCs that overexpress GSK-3β by gene transfection have significantly improved cardioprotective properties compared with MSCs alone [104]. Similarly, MSCs that overexpress the surviving gene conferred superior recovery in a rat myocardial infarction model [105]. In a rapidly advancing research field of biomaterial engineering, it has been shown that 3-dimensional support systems that mimic the microenvironment of the ECM support markedly improved cell survival after transplantation. Such engineered scaffolds are being tested to deliver sheets of supportive cells to the myocardium and islets for treating diabetes and bone reconstruction [106, 107].

In summary multiple pretreatment strategies have been developed to enhance the performance of MSCs as ex vivo reagents for angiogenesis and tissue repair. To our knowledge none have been tested clinically but preclinical results indicate efficacy and safety of most protocols and support clinical translation. The known roles of MSCs in promoting angiogenesis are summarized in Figure 2.

4. Functional Enhancement of MSCs by GHRH Agonists

4.1. Expression of GHRH Receptor on MSCs. In addition to the hypothalamus and pituitary, GHRH receptors are expressed in multiple extrahypothalamic tissues including

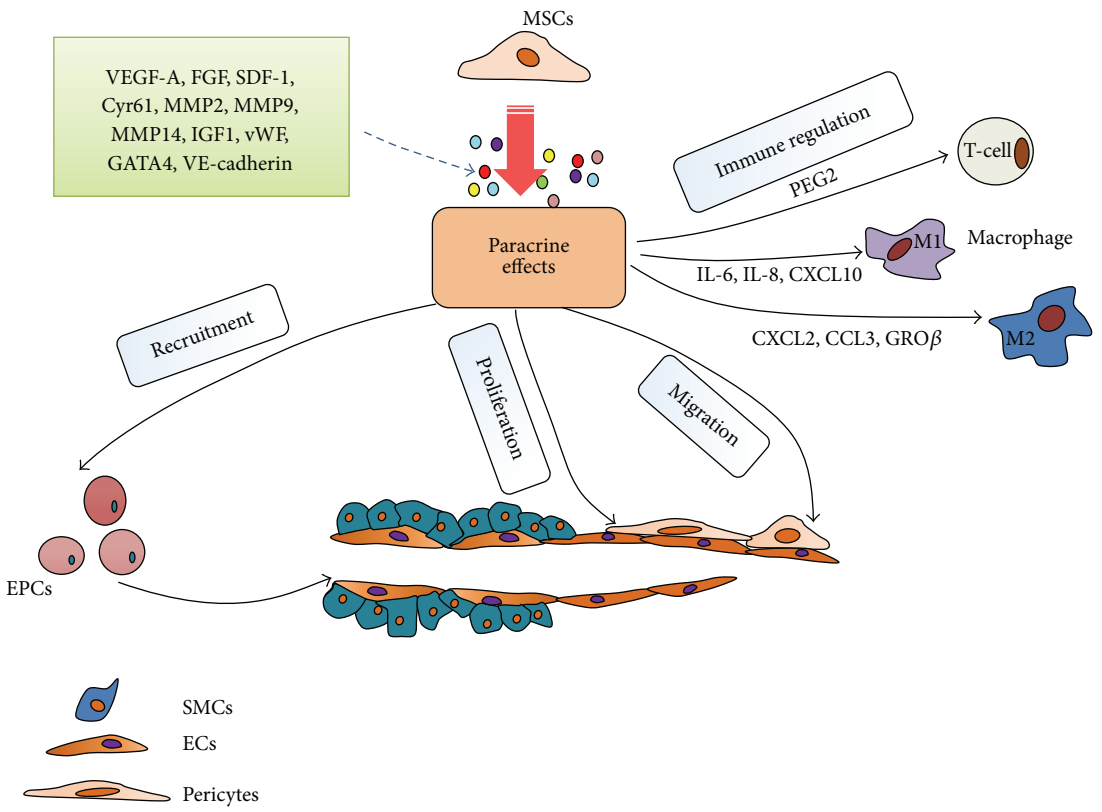

Figure 2: Proangiogenic roles of MSCs. MSCs promote angiogenesis by (1) secreting proangiogenic bioactive factors; (2) functioning as pericytes to support EC proliferation and maturation; (3) recruiting EPCs and other progenitor cells into neoblood vessels; (4) regulating the immune response of the microenvironment by modulating recruitment of immune cells.

renal medulla, renal pelvis, heart, lung, small intestine, and testis [108, 109]. Our laboratory and others have shown that GHRH receptors are also expressed on MSCs [20, 110].

4.2. GHRH Analogue Exerts Similar Effect as Proangiogenesis Ability of MSCs. GHRH and its analogues promote cell proliferation in multiple target tissues by binding and activating the GHRH receptor. GHRH analogues may have improved therapeutic properties compared with the parent GHRH. GHRH analogues have been shown to augment VEGF-A secretion in the contexts of neuroendocrine tumor cells and myocardial infarction [1, 2]. It was recently shown that treatment of MSCs from S-nitrosoglutathione reductase (GSNOR) knockout mice with a GHRH analogue JI-38 enhanced VEGF production and stimulated the angiogenic potential of JI-38-treated MSCs [20]. JI-38 appears to stimulate a paracrine circuit by binding to GHRH-R on MSCs promoting VEGF secretion and proangiogenesis.

4.3. Regulation of MSC Proliferation by GHRH Agonists. In one of its classical pathways, GHRH stimulates cell cycle entry and proliferation via activating adenylate cyclase and subsequently stimulating PKA to increase influx of Ca^{2+} through plasma membranes [111, 112]. GHRH stimulation also activates phospholipase C (PLC), increasing the production of inositol phosphates (IP) and diacylglycerol (DAG) and activating PKC in pituitary somatotrophs [113]. The same signaling pathways may cause enhanced proliferation by GHRH agonists and other factors when GHRH-Rs are activated in MSCs. Previous studies confirmed classic signal activation by cAMP and PKA in MSCs. In addition, a new factor, Epac1, was discovered as an exchange protein activated by cAMP, which leads to the activation of Ras-related protein 1 (Rap1) to further trigger the Akt phosphorylation. Activated (p)-Akt is a central regulator of insulin and IGF-1 signaling and can promote translocation of β-catenin to nucleus to augment the expression of c-myc and VEGF thereby controlling MSC proliferation [114].

As discussed earlier in this review, GHRH and its agonists promote the proliferation of pituitary cells through the activation of ERK1/2 and JAK/STAT3 signaling pathways [115]. These pathways are also required for GHRH to activate MSC proliferation. Indeed, the canonical mitogen-activated protein kinase (MAPK) signaling systems are well known to exert major control over cell metabolism, growth, differentiation, and cell death/survival pathways. Jaiswal et al. recently reported that MAPK is centrally involved in the process of proliferation and differentiation of human MSCs into osteogenic lineage [116]. GHRH agonists and analogues are likely to use to the same pathways to stimulate MSC proliferation.

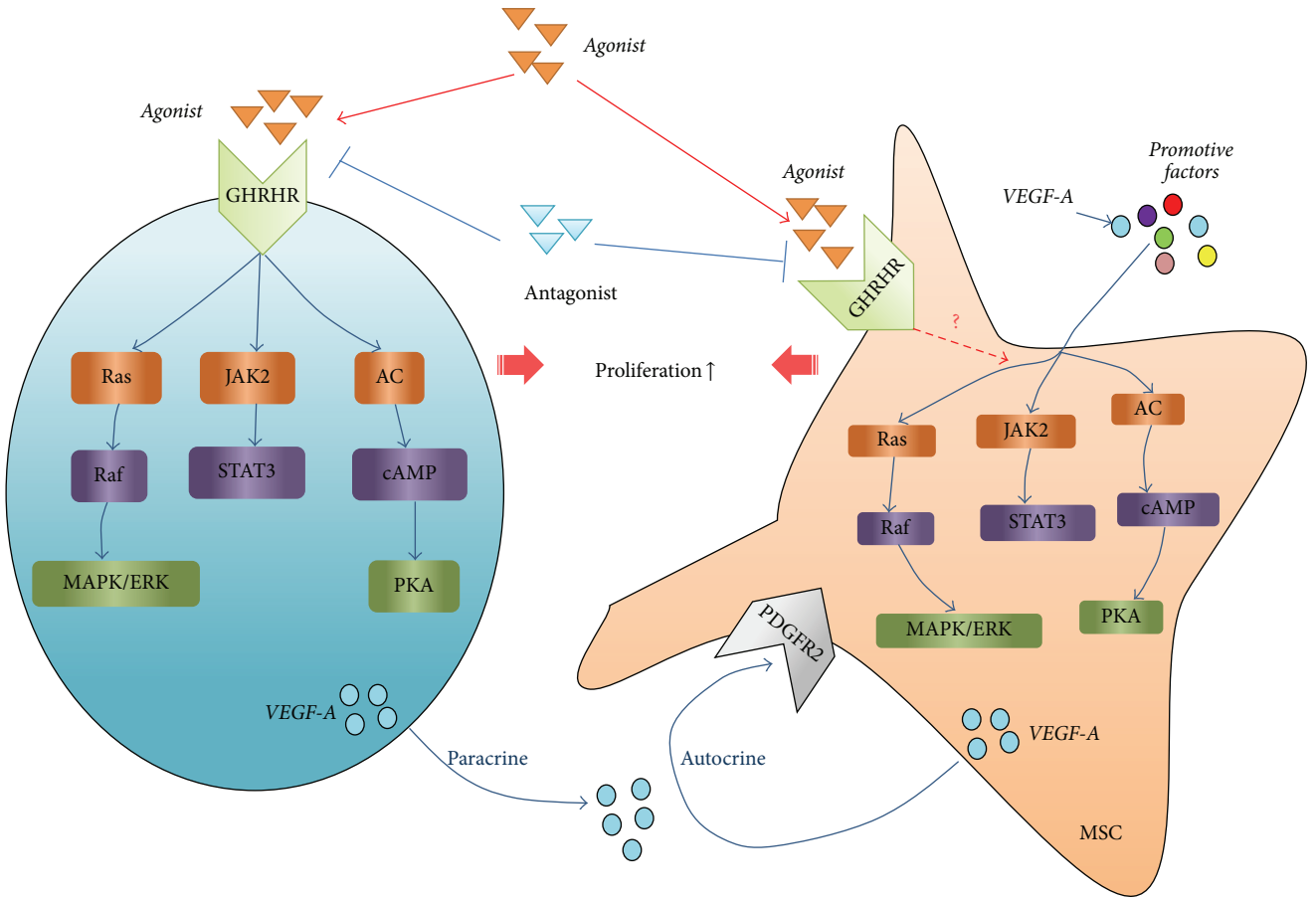

Figure 3: Effects of GHRH agonists on MSCs. Multiple cell types including MSCs express the GHRH receptor and can respond to GHRH agonists and antagonists. Receptor activation communicates with diverse survival pathways that transmit paracrine and autocrine signals to promote cytoprotection, antiapoptosis, and angiogenesis.

4.4. Antiproliferative Role of GHRH Antagonists on Neoplasms. GHRH and its receptor have been found in many tumor cell lines and neoplasms [111, 117–119]. GHRH can stimulate tumor growth through the GHRH/GH/IGF-1 axis and local GHRH stimulatory loops between GHRH and its receptor. By obstructing the interaction of GHRH with its receptor using an GHRH antagonist, it should be possible to prevent or reduce tumor cell hyperplasia by this pathway [1]. Molecules known to play important roles in cell proliferation including cAMP, PKC, and p21 have been shown to be the effective inhibited targets of GHRH antagonists for their antiproliferation and tumor suppressive actions [37, 120, 121]. These signaling intermediates also regulate MSCs proliferation.

4.5. Direct Effect of GHRH Agonists on MSCs. Treatment of MSCs with GHRH agonist JI-34 was shown to significantly increase VEGF expression in human MSCs [20]. Recently we reported that pretreatment of MSCs with GHRH agonist JI-38 stimulated cell proliferation, ameliorated apoptosis caused by hypoxia and serum deprivation, and enhanced the proangiogenesis conferred when pretreated cells were injected into ischemic hindlimbs of mice [110]. This is the first report to implicate direct interactions between an GHRH agonist and MSCs. In another report, pretreatment of ckit+ cardiac stem cells with JI-38 also conferred significant cytoprotection that was attributed to activation of ERK and Akt survival pathway [122]. Similar pathways may mediate the effects of JI-38 on MSCs described by our group. The avenues whereby GHRH agonists may enhance cytoprotection and angiogenesis are summarized in Figure 3.

5. Clinical Prospects

So far GHRH analogues have shown promise in preclinical studies. For other hypothalamic hormones, many clinical applications already exist. As early as 1971, Schally et al. isolated luteinizing hormone-releasing hormone (LH-RH) and later introduced analogues of LH-RH into clinical use as antitumor drugs [123]. These studies were extended to advanced endocrine therapy for hormone-related diseases. Schally's team also were the first to describe GHRH antagonists and their possible application for cancer treatment [124]. The recent demonstrations by our group and others, respectively, that JI-38 markedly enhanced the functional properties and potential therapy by MSCs and cardiac stem cells in different models of ischemia raise the possibility that

GHRH agonists may be useful agents to augment clinical cell therapy. At the present time MSCs or cardiac stem cells are the leading candidates for such therapy. Because multiple approaches have been used for "precondition" it will be important to compare safety and efficacy side by side in the same study to determine the optimal conditions for clinical trials.

6. Conclusion

GHRH and its analogues regulate cell growth, proliferation, differentiation, and survival through complex networks of signaling pathways involving GHRH/GH/IGF-1 and/or local activation of GHRH receptors on extrapituitary systems and paracrine/autocrine loops. Agonists of GHRH can induce a prolonged stimulation of growth and proliferation via the cAMP/PKA and ERK and JAK2/STAT3 signaling pathways, while the antagonists of GHRH block these effects by competitively binding to the receptor. Regulation by GHRH analogues of the proliferation of normal tissues and neoplasms provides for promising approaches to clinical treatments directed at tissue repair and antitumor treatments.

MSCs are leading candidates for tissue repair and angiogenesis. The superiority of MSCs is attributed to their pluripotency, immune-privilege, and rich factories of secreted paracrine factors that enhance cell survival and confer cytoprotection and angiogenesis. GHRH agonists are one of several potential candidates that confer a powerful "preconditioning" effect to MSCs that enhances viability, mobility, and therapeutic potential. Through the activation of GHRH receptors on the surface of MSCs, GHRH agonists exert cytoprotection of MSCs and confer enhanced angiogenesis. The current results warrant further studies to compare different preconditioning strategies and begin the process of translating the most promising to clinical trials.

Competing Interests

The authors declare that no competing financial interests exist.

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Review Article

Endothelial Progenitor Cells in Diabetic Microvascular Complications: Friends or Foes?

Cai-Guo Yu,[1,2] Ning Zhang,[1,2] Sha-Sha Yuan,[1,2] Yan Ma,[1,2] Long-Yan Yang,[1,2] Ying-Mei Feng,[1,2] and Dong Zhao[1,2]

[1]*Beijing Key Laboratory of Diabetes Prevention and Research, Luhe Hospital, Capital Medical University, Beijing 101149, China*
[2]*Department of Endocrinology, Luhe Hospital, Capital Medical University, Beijing 101149, China*

Correspondence should be addressed to Dong Zhao; zdoc66@126.com

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Despite being featured as metabolic disorder, diabetic patients are largely affected by hyperglycemia-induced vascular abnormality. Accumulated evidence has confirmed the beneficial effect of endothelial progenitor cells (EPCs) in coronary heart disease. However, antivascular endothelial growth factor (anti-VEGF) treatment is the main therapy for diabetic retinopathy and nephropathy, indicating the uncertain role of EPCs in the pathogenesis of diabetic microvascular disease. In this review, we first illustrate how hyperglycemia induces metabolic and epigenetic changes in EPCs, which exerts deleterious impact on their number and function. We then discuss how abnormal angiogenesis develops in eyes and kidneys under diabetes condition, focusing on "VEGF uncoupling with nitric oxide" and "competitive angiopoietin 1/angiopoietin 2" mechanisms that are shared in both organs. Next, we dissect the nature of EPCs in diabetic microvascular complications. After we overview the current EPCs-related strategies, we point out new EPCs-associated options for future exploration. Ultimately, we hope that this review would uncover the mysterious nature of EPCs in diabetic microvascular disease for therapeutics.

1. Introduction

Prelude. Diabetes is a type of metabolic disorder, featured as insulin resistance and insufficient insulin release due to pancreatic β cell dysfunction. Hyperglycemia appears in the early stage of diabetes. As the disease progresses, patients display excess thirst (polydipsia), frequent urination (polyuria), increased hunger (polyphagia), and loss of body weight. As most pathological changes involved in blood vessels of multiple organs, macro- and microvascular complications are frequently observed in diabetic patients and become the major cause of mortality.

Endothelial progenitor cells (EPCs) were first described nearly two decades ago. They participate in endothelial repair either by secreting angiogenic factors or by incorporating into disrupted endothelium and differentiating into endothelial cells to maintain endothelium integrity. Despite the long-term debate on the nature and identification of EPCs, compelling data showed that EPCs improved blood perfusion in peripheral ischemia. Nevertheless, abnormal angiogenesis is the featured pathological hallmark in diabetic retinopathy and nephropathy and, therefore, anti-VEGF treatment has been applied for treating the microvascular abnormality. Thus, the questions are rising: what is the nature of EPCs in diabetic microvascular disease? Could we apply EPCs for the treatment of diabetic retinopathy and nephropathy?

The Presence of EPCs in Nature. EPCs were first described in 1997. When $CD34^+$ cells were isolated from human peripheral blood, they could differentiate into endothelial cells *in vitro* and participate in angiogenesis *in vivo* [1]. Despite a long debate about EPC identity, more and more data collectively indicated the presence of EPCs in nature: (1) human induced pluripotent cells (hiPSCs) could differentiate into vascular endothelial progenitors that could incorporate into injured endothelium *in vivo* [2, 3]; (2) despite being putative, both adult and human embryonic stem cells-derived hemangioblasts have shown endothelial capacities [4]; (3)

different mechanical cues could sense cardiosphere-derived cells with enriched cKit$^+$ subpopulation to differentiate either to endothelial or to cardiomyogenic lineage [5]; (4) Prox-1$^+$ cells emerging at E9.5 could sprout from the veins to form lymph sacs and an initial lymphatic vasculature [6].

Heterogeneous EPCs Population. Data from different groups consistently demonstrated that EPCs are heterogeneous populations and classified into early EPCs and late EPCs. Early EPCs, also known as colony-forming unit-ECs (CFU-ECs) or CFU-Hill, exhibited a spindle shaped morphology, had poor proliferative capacity, and produced to a high extent angiogenic cytokines [1, 7]. By contrast, late EPCs, now generally termed as endothelial colony-forming cells (ECFCs), showed a cobblestone-shaped morphology and highly proliferative activity when cultured *in vitro*. In response to injury, they could be mobilized from bone marrow or other locations, migrate toward lesion site, and incorporate into injured endothelium *in vivo* [8–12]. Therefore, ECFCs are the main target under investigation.

Identification and Cultivation of EPCs. So far, no unique surface marker has been identified for EPCs. Instead, different combinations of surface markers have been used for EPCs identification, such as CD34$^+$/VEGFR2$^+$ and CD133$^+$/VE-cadherin$^+$ [13–16]. Although these markers could help us to quantify EPCs *in vivo*, it is yet uncertain whether the cells isolated from these markers could give rise to ECFCs *in vitro*. Thus, functional assays become more reliable for EPCs confirmation, which include their morphology, ability to form vasculature *in vitro*, and incorporation into vasculature upon injection *in vivo*.

With regard to cultivation, early EPCs could be obtained from mononuclear cells (MNCs) of human peripheral blood or cord blood after 4-5 days of culture in medium containing endothelial growth factors and fetal calf serum [7, 17]. They are recognized by monocytic morphology, uptake of acetylated low-density lipoprotein (AcLDL), and binding to lectin.

To obtain ECFCs, mononuclear cells are cultured and passaged in endothelial cell specific medium with endothelial growth factors for at least 28 days. During culture, endothelial colonies appear and can be picked up for further expansion [16]. As the development of stem cell technology, embryonic stem cells and induced pluripotent stem cells (iPSCs) have become useful alternative cell sources to generate EPCs for practice [2, 3, 18, 19].

Reduced Number and Impaired Function of EPCs in Diabetes. Besides their huge capacity for endothelium generation and maintenance of endothelium integrity, a recent observation indicated the beneficial effect of EPCs on β cell survival. When pancreatic β cells were cotransplanted with EPCs, a better β cell engraftment with preserved function was observed, resulting in improved cure rate and initial glycemic control [20]. Unfortunately, EPCs number was significantly reduced with impaired function in diabetic patients as well as db/db mice, which was associated with poor vascular outcome in diabetes [21, 22]. In the next section, we discuss how hyperglycemia induces metabolic and epigenetic changes in EPCs.

2. Metabolic and Epigenetic Change of EPCs in Diabetes

2.1. Hyperglycemia-Associated Metabolic Change. Hyperglycemia induces advanced glycated end products (AGEs) formation and oxidative stress and increases reactive oxygen species (ROS) production in mitochondrion, which are the main killers of EPCs apoptosis [23]. Increased ROS production could also stimulate AGEs production, which further triggers ROS production. To make it worse, both of them synergistically activate nuclear factor-kappa B (NF-κB) transcription [24]. NF-κB is a well-known transcription factor with most of its target genes encoding inflammatory proteins inducing interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) and p53 as well as inducible nitric oxide synthase (iNOS). Thus, a loop between iNOS, ROS, NF-κB, and AGEs develops to aggravate inflammation cascade [25–27]. Apart from ROS production in mitochondrion, high concentration of glucose provokes endoplasmic reticulum stress (ER stress), which promotes EPCs apoptosis and reduces EPCs migratory function [28]. Autophagy is a homeostatic process and is involved in organelle recycling and protein degradation. However, in response to high glucose-induced ER stress and oxidative stress, excessive autophagy might contribute to EPCs death [29].

In line with increased apoptosis by hyperglycemia, high level of glucose jeopardizes EPCs proliferative capacity partially via its inhibition of Akt phosphorylation and subsequently NOS activation or via activation of C-Jun N-terminal kinase (JNK) pathway [30–32]. High glucose also induces EPCs senescence by one of NF-κB target genes, p53, and activation of p38MAPK pathway [33]. Figure 1 summarizes the molecular mechanisms of how hyperglycemia adversely affected EPCs number and function.

2.2. Hyperglycemia-Associated Epigenetic Change. Glycemic control is the initial treatment for diabetes. Nonetheless, tight glycemic control in the late stage of the disease fails to attenuate diabetic vascular complications. This phenomenon of glycemic memory was noticed in 1987 [34] and was extensively validated by different diabetic animal models and preclinical findings. These reports consistently pointed out that intensive glycemic control in the early stage could delay the development of diabetic microvascular abnormalities in type-1 [35] and type-2 diabetic subjects [36, 37] as well as streptozotocin-induced diabetic rats [38].

Glycemic memory is defined as the inexorable progression of diabetic vascular complication that is linked to uncontrolled hyperglycemia in the early stage of diabetes despite a tight glycemic control in the follow-on period. How does glycemic memory come? *In vitro* data have demonstrated that exposure of aortic endothelial cells to high glucose for 16 hours promotes NF-κB p65 gene transcription. This transcription activation is sustained even when endothelial cells are cultivated back to normal glucose concentration [39]. Similarly, when Zheng et al. challenged retinal endothelial cells with high glucose conditions for one week and then returned to normal glucose conditions for two weeks, they noticed that one week of hyperglycemia was sufficient to

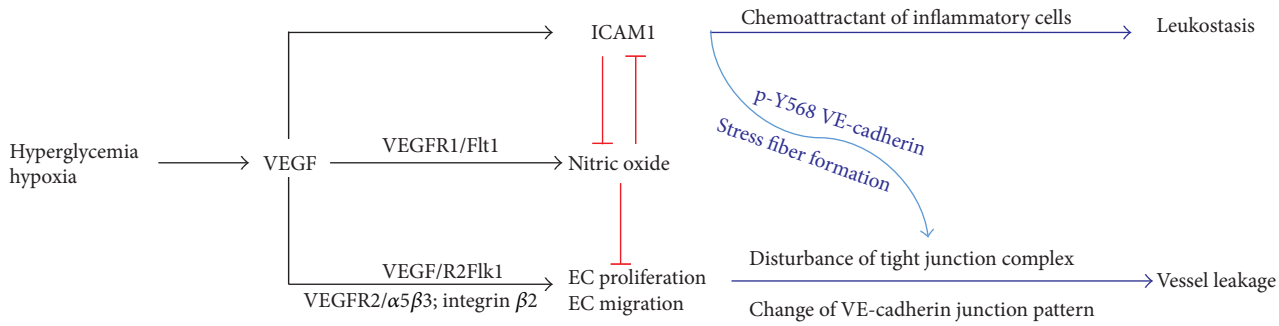

Figure 1: Uncoupling VEGF with nitric oxide in microvascular endothelial cells. Hyperglycemia promoted local VEGF production. VEGF stimulated endothelial cell proliferation via binding to VEGFR2. It induced endothelial cell migration and altered junction complex, in particular, VE-cadherin on the membrane. In parallel, VEGF promoted ICAM1 expression in endothelial cells which triggered inflammatory response to attack endothelium and made VE-cadherin dissemble. ICAM1 could also enhance ROS production that has negative impact on nitric oxide level and bioavailability. Although VEGF promoted nitric oxide production in endothelial cells via its receptor Flt1, nitric oxide is significantly reduced by ROS and oxidative stress, losing its inhibitory effect on VEGF-induced endothelial cell proliferation, migration, and activation.

induce NF-κB activation which remained unchanged for the remaining two weeks [40]. They further found that Sirtuin 1 (SIRT1) was the main regulator in the event. Belonging to a class 3 HDAC, SIRT1 deacetylates H3K14 and H14K16 to control ROS production in endothelial cells and positively regulates EPCs differentiation into endothelial lineage [41].

Epigenetic modulation mainly includes posttranslational histone modification, DNA methylation, and microRNA-regulated transcriptional changes. As mentioned above, hyperglycemia-induced oxidative stress, ROS, and AGEs are the main factors for EPCs apoptosis and dysfunction. They are also potent inducers for epigenetic changes in EPCs. For instance, ROS is associated with a series of histone modifications in the promoter and enhancer of superoxide dismutase 2 (SOD2) gene in rat retinal endothelial cells [42]. High glucose triggers the increase of H3K4mel but decreases H3K9me2 and H3K9me3 expression level at the promoter of NF-κB in human microvascular endothelial cells, leading to NF-κB activation [43]. Furthermore, the histone codes H3K9ac, H3K12ac, H3K4me2, and H3K4me3 suppress eNOS transcription, leading to decreased nitric oxide [44]. All these epigenetic modifications accelerate proinflammatory machinery for EPCs apoptosis and loss of function.

It is well accepted that EPCs-mediated endothelium repair is beneficial for coronary heart disease, whereas abnormal angiogenesis is the hallmark of diabetic retinopathy and nephropathy and anti-VEGF agents have been applied for treating diabetic microvascular diseases. Thus, the question is coming: how do EPCs participate in the microvascular disease? Next, we first review how abnormal angiogenesis initiates diabetic microvascular disease.

3. Abnormal Angiogenesis in Diabetic Retinopathy and Nephropathy

Diabetic retinopathy is one of most frequent complications in diabetes, which is the leading cause of vision loss. After 20 years of diabetes, almost all type-1 diabetes patients, 80% of insulin-dependent diabetics, and 50% of insulin-independent type-2 diabetic patients will develop retinopathy [45, 46]. Diabetic retinopathy is traditionally classified into two main clinical forms: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR), based on the presence or absence of neovascularization.

In a similar situation to diabetic nephropathy, 20%–40% of diabetic patients develop nephropathy [47], which is featured as initial microalbuminuria and then followed by a lot of albuminuria and increased serum creatinine level. Diabetic nephropathy has become the leading cause of end stage renal disease worldwide.

From the pathological view of diabetic retinopathy and nephropathy, aberrant angiogenesis is the common feature in the diseases, which is characterized as hypoxia-induced local VEGF expression, reducing nitric oxide level and availability, oxidative stress, vascular leakage, and inflammation. In parallel with VEGF axis, the imbalanced expression between angiotensin I and angiotensin II serves as another mechanism for endothelial dysfunction and hyperpermeability in diabetic eyes and kidneys.

3.1. Uncoupling VEGF with Nitric Oxide. Endothelium acts as physiological barrier between serum proteins and blood vessel, whose integrity is tightly controlled by nitric oxide produced by endothelial cells. Physical level of VEGF promotes endothelial cell proliferation via its receptor VEGFR2 and stimulates eNOS activation for nitric oxide (NO) production via VEGFR1.

In the setting of diabetes, hypoxia-induced VEGF production acts on endothelial cells for proliferation, migration, and NO production; in the meantime, it stimulates intercellular cell adhesion molecule-1 (ICAM1) expression on the surface of endothelial cells [48, 49]. ICAM1 triggers NADPH oxidase activation for reactive oxygen species (ROS) production [48]. ROS, advanced glycation end products (AGEs), asymmetric dimethylarginine (ADMA), and hyperglycemia dramatically increase arginase activity [50]. Arginase competes with NOS for the common substrate, L-arginine, resulting in insufficient substrate for NOS. Uncoupled NOS in turn

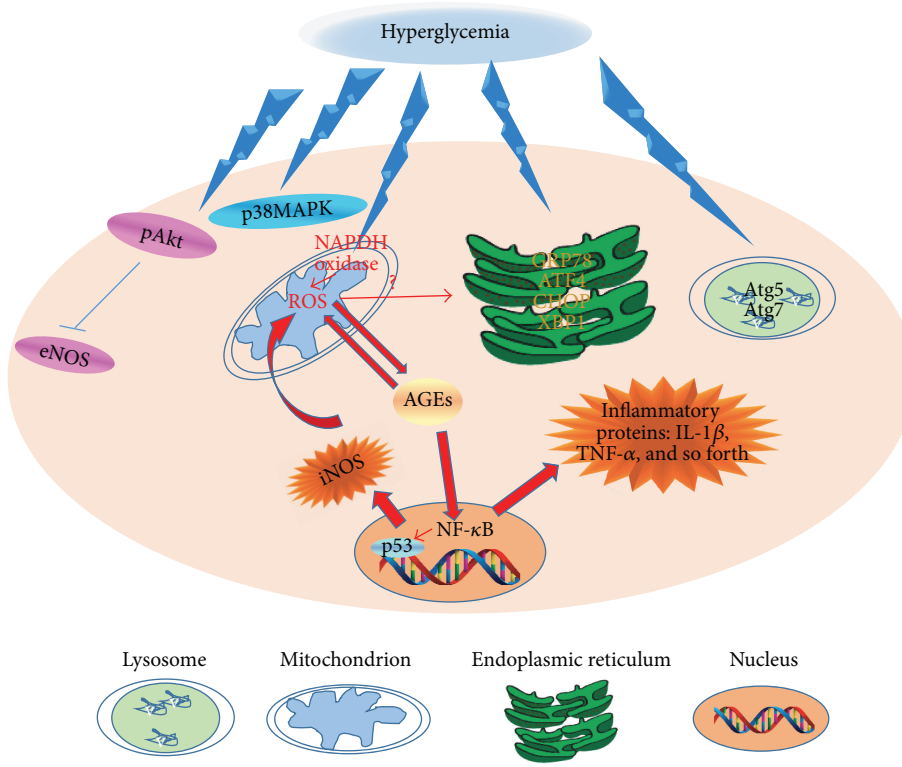

Figure 2: The detrimental effect of hyperglycemia on endothelial progenitor cells (EPCs) number and function. High level of glucose stimulates ROS production through activation of NAPDH oxidase. Increased reactive oxygen species (ROS) production triggers advanced glycated end products (AGEs) formation. AGE in turn further increases ROS production and, in the meantime, promotes nuclear factor-kappa B (NF-κB) transcription. NF-κB is crucially involved in inflammation via transcriptional activation of its target genes such as IL-1β and TNF-α. In parallel, NF-κB also activates p53 to accelerate cell senescence and iNOS that further potentiates ROS production. Except mitochondrial diabetic retinopathy ion damage,hyperglycemia induces endoplasmic reticulum (ER) stress and excessive autophagy to further facilitate EPCs death. Apart from that, hyperglycemia inhibits Akt phosphorylation and subsequently eNOS activation. It also activates p38MAPK pathway to promote cell death and senescence. Ultimately, EPCs are triggered to undergo apoptosis and become dysfunctional.

use more oxygen molecules to generate superoxide, which catabolizes any available NO for peroxynitrite formation [50]. In addition, ADMA is an endogenous inhibitor of eNOS but its level is increased in diabetes [51]. Ultimately, NO level and availability are severely reduced.

In contrast to diminished NO availability, VEGF induces abnormal endothelial cell proliferation via VEGFR2 and, more importantly, enhances migration but disrupts cytoskeleton rearrangement through cross talk between VEGFR2 and integrin $\alpha5\beta3$ or integrin $\beta2$, separately [52, 53]. Moreover, under physical angiogenesis, Notch/VEGFR2 modulates differential dynamics of VE-cadherin junction pattern during sprouting. When switched to pathological high VEGF condition, the differential VE-cadherin mobility is lost and thus tip cell competition and stalk cell intercalation are disturbed [54]. In parallel, downstream of VEGF-induced ICAM1 expression and ROS production, Src kinase and protein tyrosine kinase 2β are activated, both of which phosphorylate Y-658 on VE-cadherin for dissembling this protein [55]. Moreover, ICAM1 could also activate Rho GTPase for stress fiber formation, leading to permeability [55]. Nitric oxide antagonizes endothelial cell proliferation and inflammation, thereby maintaining endothelium integrity [56].

The pathological pattern "VEGF uncoupling with NO" is preserved and serves as the main mechanism in diabetic retinopathy and nephrology [52, 57]. For instance, studies from diabetic eNOS knockout mice have demonstrated that this mouse model develops severe albuminuria as well as increased VEGF expression in the kidney. Histological analysis confirms diabetic nephropathy in this model as evidenced by mesangial expansion, glomerular basement membrane thickening, and mesangiolysis [57]. Concerning the eye, VEGF level has been found to be significantly increased in ocular tissues in diabetic patients with retinopathy, which is accompanied with inflammation and uncoupled eNOS [58, 59].

Taken together, irregular endothelial proliferation, migration, cytoskeleton rearrangement, and dissembled VE-cadherin contribute to vessel leakage. The "uncoupling of VEGF with NO" pattern is shown in Figure 2.

3.2. Angiopoietins in Diabetic Eye and Kidney. On top of "VEGF uncoupling with NO," angiopoietin 1 (Angl)/Tie2 is another system that protects endothelium integrity. Upon binding to Tie2 tyrosine kinase receptor, Angl has been

shown to reduce endothelium permeability, suppress NF-κB-associated inflammation, and antagonize VEGF functions. In contrast, Ang2 is the endogenous antagonist of Ang1. The increase of Ang2 in diabetes condition competes with Ang1 for binding to Tie2, rendering the Ang1-regulated antiangiogenesis toward Ang2-mediated abnormal angiogenesis [60]. Recent study reported that VEGF and Ang1 exert opposing effect on endothelial cell permeability via their distinct modulation of RhoA-specific guanine nucleotide exchange factor (Syx). In the study, the authors elaborately showed that Syx was recruited to endothelial junction by Mupp1 and formed a complex with multiple members of the apicobasal polarity complexes (CRB) on the membrane for junction stabilization. They further demonstrated that VEGF caused Syx dissociation from Mupp1 and Syx translocation from cell junctions, resulting in junction disassembly [61].

A potent Ang1 variant, cartilage oligomeric matrix protein (COMP), was developed nearly one decade ago. Administration of COMP-Ang1 reverses hyperglycemia-induced kidney dysfunction by suppression of ICAM1 and monocyte chemoattractant protein-1 and monocyte/macrophage infiltration in diabetic db/db mice [60]. COMP-Ang1 also reduces renal tissue levels of transforming growth factor-beta1 (TGF-β1), alpha-smooth muscle actin, and fibronectin, as well as Smad 2/3 expression, but increases Smad 7 expression [60]. Likewise, recent data demonstrate that COMP-Ang1 could ameliorate retinopathy and stabilize blood retinal barrier in diabetic Ins2Akita mice [63].

4. EPCs in Diabetic Retinopathy and Nephropathy

4.1. EPCs in Diabetic Retinopathy. After observing the presence of abnormal angiogenesis in the development of diabetic retinopathy, antiangiogenesis therapies such as anti-VEGF agents have been taken to treat diabetic patients with retinopathy. This has brought a long debate: are EPCs good or bad in the disease? There is no definite answer so far. As the complicated pathogenesis and different types of diabetic retinopathy, either decreased or increased or unchanged EPCs number has been reported in diabetic patients with severe retinopathy when comparing to diabetic patients with no to mild retinopathy or healthy subjects [64–68].

To be noted, in the studies where they found increased EPCs number in the patients with diabetic retinopathy, EPCs function such as migration and mobilization and homing was often impaired. And this EPCs pattern, that is, increased EPCs number with impaired function, is consistently conserved in both type-1 and type-2 diabetic patients [68, 69]. Paradoxically, intravitreal delivery of COMP-Ang1 improves endothelial integrity and ameliorates vascular leakage by promoting the incorporation of endothelial colony-forming cells into retinal vasculature [63] in diabetic mice. Giving the nature of ECFCs in endothelial regeneration and NO production, this is an excellent example illustrating using the right ECFCs in reversing diabetic retinopathy.

4.2. EPCs in Diabetic Nephropathy. The early pathological features of diabetic nephropathy include hyperperfusion and hyperfiltration due to endothelial cell damage and abnormal angiogenesis. As the inflammation becomes more severe, glomeruli fibrosis develops, resulting in kidney failure. Although the exact mechanisms of nephropathy are not fully understood, AGEs, oxidative stress, and the activation of the renin-angiotensin-aldosterone system (RAAS) facilitate and strengthen these changes partially through activation of TGF-β1 signaling and increased vascular endothelial growth factor (VEGF) expression in the kidney toward progression of fibrosis and renal failure [70–72]. Therefore, antagonism of VEGF signaling using anti-VEGF antibody or endogenous inhibitor of VEGF or inhibition of VEGF receptor-1 phosphorylation has been used to improve early renal function in diabetic rats injected with STZ or db/db mice [73–75]. In parallel, inhibition of AGEs suppresses TGF-β1 and VEGF signaling pathways and alleviated diabetic nephropathy [72].

As endothelial cell damage occurs in the early stage of kidney dysfunction, its repair is not well processed due to EPCs defect in diabetes. In line with this, Makino and coworkers reported the negative correlation between EPCs number and microalbuminuria or albumin excretion rate in both type-1 and type-2 diabetic patients, respectively [76, 77], suggesting the protective effect of EPCs in the structure and function of glomeruli.

Putting these evidences together, ECFCs could be a promising target for treating diabetic retinopathy and nephropathy. Giving that ECFCs number and function are reduced in diabetic condition, how to obtain sufficient amount of ECFCs with desirable function for therapeutic is under investigation.

5. ECFC Therapy for Diabetic Microvascular Disease

5.1. Drugs. Till present, some of the antidiabetic drugs like metformin, thiazolidinediones, GLP-1 agonists, DPP-4 inhibitors, and insulin might increase EPCs number and improve EPCs function with increase of nitric oxide bioavailability [78–87]. Except for these antidiabetic drugs, lipid-lowering drugs, statins, improve EPCs number and function [88]. Table 1 provides an overview of these antidiabetic drugs in the aspects of their effects on EPCs number and function.

5.2. Cord- and Cord Blood-Derived ECFCs. For clinical practice, ECFCs can be obtained from long-term cultivation of mononuclear cells isolated from blood. Nevertheless, the quality and functionality of ECFCs could vary from one group to another, which could be due to the different passages and ECFCs purity they use and risk factors that the donors carry on [89].

Alternatively, induced pluripotent stem cells (iPSCs) generated from $CD34^+$ cord blood cells have shown huge capability of differentiating into ECFCs. For therapeutic purposes, autologous iPSCs are more favored to avoid immune rejection. However, the experimental practice of iPSC generated from patients is always frustrating by growth arrest,

Table 1: Overview of the effect of antidiabetic drugs on EPCs number and function.

Author	Drug	Groups	Conclusion
Sorrentino et al., 2007	Thiazolidinedione (TZD)	T2DM patients T2DM: $n = 30$ Controls: $n = 10$	Rosiglitazone restored nitric oxide bioavailability and improved EPC function
Spigoni et al., 2012	Thiazolidinedione (TZD)	*In vitro* cultured ECFC isolated from subjects with impaired glucose tolerance: $n = 14$	Pioglitazone improved ECFC viability and capacity to form tubular-like structures
Humpert et al., 2008	Insulin	ECFC culture, *in vitro*	Insulin improved EPC function
Fadini et al., 2011	Insulin	T2DM patients: $n = 42$	Insulin increased EPC count
Maiorino et al., 2016	Insulin	T1DM patients insulin infusion: $n = 41$	Insulin infusion increased EPC number in T1DM patients
Liao et al., 2010	Metformin	Newly diagnosed T2DM: $n = 46$ Non-DM: $n = 51$	Metformin increased EPC number in the blood
Liu et al., 2011 [62]	GLP-1 agonists	EPC culture, *in vitro*	GLP-1 enhanced EPC proliferation and VEGF production in EPC
Gonçalves et al., 2012	DPP-4 inhibitors	Diabetic rats	Sitagliptin increased the number of $CD34^+$ cells in the blood
Mohler III et al., 2009	Atorvastatin; ezetimibe	Diabetic swines	EPC number in the circulation was increased by atorvastatin
Chang et al., 2010	Adiponectin	Adiponectin deficient db/db mice	Adiponectin rescued EPC senescence

uncontrolled differentiation, and incomplete function [90, 91]. Recent studies shed light on getting desired ECFCs. After iPSCs were obtained from healthy donors and patients with type-1 diabetes, the differentiation of iPSCs toward vascular cells was processed in an adherent, feeder-free differentiation protocol and further assembled in 3D engineered hyaluronic acid hydrogels for maturation. When injecting, the yielded endothelial progenitors were incorporated into vasculature [92]. In line with this study, Park et al. reported that vascular progenitors generated from human iPSCs derived from cord blood possessed greater capacity for homing and long-term incorporation into injured retinal vessels [2]. These studies establish promising strategies for applying iPSC-derived endothelial progenitors for stabilizing microvascular structure and inhibiting vessel leakage.

5.3. Genetically Modified ECFC. When dissecting the two main pathological machineries that affect retinopathy and nephropathy, applying ECFC with higher level of nitric oxide or Angl would be favorable for stabilized capillaries by reversing "uncoupled VEGF with nitric oxide," balancing "Angl/Ang2 competition" and "rendering Angl/VEGF." This idea awaits future evaluation.

Additional Points

Endothelial progenitors play a major role in the maintenance of endothelium integrity. Unfortunately, hyperglycemia induces metabolic and epigenetic changes in EPCs, leading to EPC apoptosis and reduced function. Reduced EPCs number and function jeopardize endothelial regeneration, resulting in hyperpermeability and inflammation in capillaries and then development of retinopathy and nephropathy. Providing the angiogenesis potential of incorporating into injured vessel and increased nitric oxide production upon stimulation, ECFCs might become promising and complimentary therapeutic targets for treating diabetic patients. iPSC-based ECFCs would be one of the main strategies for clinical therapeutic for diabetic microvascular abnormality. To gain intact endothelium and protect vessel from retinopathy and nephropathy, it might be advisable to administer ECFC in the early stage of diabetes for better efficacy.

Competing Interests

The authors declare no competing interests regarding the publication of this paper.

Authors' Contributions

Cai-Guo Yu, Ning Zhang, Sha-Sha Yuan, and Yan Ma contributed equally to the paper.

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Research Article

The Engrailed-1 Gene Stimulates Brown Adipogenesis

Chuanhai Zhang,[1,2] Yibing Weng,[3] Fangxiong Shi,[1] and Wanzhu Jin[2]

[1]*Laboratory of Animal Reproduction, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China*
[2]*Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*
[3]*Department of Critical Care Medicine and Emergency Room, Luhe Hospital, Capital Medical University, Beijing 101149, China*

Correspondence should be addressed to Fangxiong Shi; fxshi@njau.edu.cn and Wanzhu Jin; jinw@ioz.ac.cn

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As a thermogenic organ, brown adipose tissue (BAT) has received a great attention in treating obesity and related diseases. It has been reported that brown adipocyte was derived from engrailed-1 (EN1) positive central dermomyotome. However, functions of EN1 in brown adipogenesis are largely unknown. Here we demonstrated that EN1 overexpression increased while EN1 knockdown decreased lipid accumulation and the expressions of key adipogenic genes including PPARγ2 and C/EBPα and mitochondrial OXPHOS as well as BAT specific marker UCP1. Taken together, our findings clearly indicate that EN1 is a positive regulator of brown adipogenesis.

1. Introduction

In small mammals, brown adipose tissue (BAT) is a major tissue responsible for nonshivering thermogenesis [1]. The mitochondria of BAT uncouple large amounts of fuel oxidation from ATP for generation of heat [2]. Recently, we demonstrated that transplantation of BAT could prevent obesity development and reverses preexisting obesity [3, 4]. It has been shown that increasing BAT activity by cold exposure could reduce fat mass in human adults [5–7]. These results highlight that increased amount and activity of BAT are a promising avenue to combat obesity and its related diseases such as diabetes.

Brown adipogenesis is regulated by several transcription factors such as peroxisome proliferator-activated receptor γ 2 (PPARγ2), PR domain containing 16 (PRDM16), and CCAAT/enhancer-binding proteins (C/EBPs) [8–11]. The nuclear receptor corepressor RIP140, a ligand-dependent transcriptional repressor, also plays a crucial role in regulating the balance between energy storage and energy expenditure by repressing brown adipocyte differentiation [12–14]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and cell death activator A (CIDEA) are highly expressed in BAT which were known to regulate BAT differentiation [15, 16].

It has been demonstrated that myf5 positive progenitor cells are the origin of both brown adipocyte and myoblast [10]. In addition, engrailed-1 (EN1) positive central dermomyotome is also another source of brown adipocyte [17]. The EN1, a murine homologue of the *Drosophila* homeobox gene engrailed (En), is required for midbrain and cerebellum development and dorsal/ventral patterning of the limbs [18]. The mouse study also demonstrated that the expression of EN1 is critical in the correct development of the brain, limbs, and sternum [19]. However, the physiological function of EN1 during brown adipocytes differentiation has not been well studied.

To investigate the functional roles of EN1 in brown adipocyte, we take advantages of lentiviral mediated EN1 overexpression and/or knockdown technique to demonstrate here that EN1, indeed, promotes brown adipocyte differentiation by stimulating key adipogenic transcription factor, PPARγ2 expression.

2. Materials and Methods

2.1. Mice. Eight-week-old male C57BL/6J donor mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. Ob/Ob mice were from Nanjing Biomedical Research Institute of Nanjing University. For cold stimulation, C57BL/6J mice were placed in a cold chamber (4°C) or room temperature (RT) for up to 8 hrs with free access to food or water. For diet induced obesity (DIO) studies, 3-week-old male C57BL/6J mice from Vital River Laboratory Animal Technology were fed with either low-fat diet (LFD) or high-fat diet (HFD) for additional 8 weeks. The LFD (12450Bi) and HFD (D12492i) contain 10 kcal% fat and 60 kcal% fat, respectively (Research Diets). Mice were housed in the Office of Laboratory Animal Welfare certified animal facility with a 12-hour light/12-hour dark cycle. All animal studies were conducted with the approval of the Institutional Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences.

2.2. Lentivirus Package and Transfection. HEK293FT cells (Sci-Tech, Shanghai, China) were used in lentivirus package. The cells were maintained in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% FBS, 1x antibiotic-antimycotic solution, and 10 μM nonessential amino acid. The coding region of EN1 gene was amplified from a BAT cDNA sample and cloned into pCDH-CMV-MCS-EF1-copGFP Lentivector (System Biosciences, San Francisco, CA, USA). The shEN1 sequences were as follows: F: CCGGGTTCCAGGCAAACCGCTATATCTCGAGATATAGCGGTTTGCCTGGAACTTTTTG, R: AATTCAAAAAGTTCCAGGCAAACCGCTATATCTCGAGATATAGCGGTTTGCCTGGAAC and generate the sequence-verified shRNAs in pLKO.1 (Addgene plasmid # 10878). The shuttle plasmid pCDH-CMV-MCS-EF1-copGFP or pLKO.1 or shEN1-pLKO.1 and lentivirus helper plasmid were cotransfected into HEK293FT cells to produce virus. Forty-eight hours after transient transfection, the fresh lentivirus containing supernatant was harvested for future use.

2.3. Flow Cytometry and Cell Sorting. The primary brown adipocytes were prepared in accordance methods with the previous publication [20]. Floating adipocytes were separated from the SVF (Stromal Vascular Fraction) by centrifugation at 300 ×g for 3 min. SVF was sequentially filtered through 70 μm filters before staining with the following antibodies for 10 min on ice: Sca-1-APC (Miltenyi Biotec, 130-093-223), CD11b-FITC (Miltenyi Biotec, 130-081-201), and CD45-PE (Miltenyi Biotec, 130-091-610). Following antibody incubation, cells were washed, centrifuged at 300 ×g for 10 min, and sorted with a BD FACS Aria (BD Biosciences, CA, USA). Data analysis was performed using BD FACS Diva software.

2.4. Cell Culture. Cell culture related products and most other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. After being infected with lentivirus for 6–12 hours, the sorted primary brown fat preadipocytes were grown until 100% in 6-well plates and then treated with brown adipogenic induction cocktails (DMEM containing 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 0.12 mM indomethacin, and 1 nM 3,3′,5-triiodo-L-thyronine (T3)) for the first two days and the medium was replaced with differentiation medium supplemented with only insulin and T3 for additional 6 days for differentiation.

2.5. Real-Time-PCR. Total RNA was isolated using the RNeasy Mini Kit. cDNA was synthesized using random hexamers (Invitrogen, Carlsbad, CA, USA) for subsequent real-time quantitative PCR analysis (ABI Prism VIIA7; Applied Biosystems Inc., Foster City, CA, USA). PCR products were detected using SYBR Green and normalized by cyclophilin expression. Primers were designed using Primer Quest (Integrated DNA Technologies, Inc., Coralville, IA, USA). Primer sequences were available upon request.

2.6. Western Blotting. Cell and tissue lysates were prepared using RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, and protease) and phosphatase inhibitor cocktail (Roche Diagnostics Co., CA, USA). Protein concentrations were measured with a BCA assay kit (Pierce Diagnostics Co., CA, USA). Protein was separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, MA, USA). Membranes were blocked in 5% skim milk in TBST (0.02 M Tris base, 0.14 M NaCl, and 0.1% Tween 20, pH 7.4) followed by incubation with primary antibodies overnight at 4°C and then incubation with secondary antibodies conjugated with HRP. Primary antibodies used in the current study are EN1 (ab108598, R&D Systems, MN, USA), PPARγ2 (#2443, Cell Signaling Technology, MA, USA), AP2 (A0232, ABclonal Biotech Co., MA, USA), UCP1 (ab155117, Abcam Co., MA, USA), PGC1α (ab54481, Abcam Co., MA, USA), OXPHOS (ab110413, Abcam Co., MA, USA), and GAPDH (#2118, Cell Signaling Technology, MA, USA). Signals were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

2.7. Oil-Red O Staining. To detect neutral lipid, cells were stained with 0.2% (w/v) Oil-Red O (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature after fixation with 4% PFA.

2.8. Statistical Analysis. The data are presented as means ± SD. Statistical significance was tested using ANOVA or Student's t-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. EN1 Is Highly Expressed in BAT Compared to WAT. To investigate the expression of EN1 in related tissues, we analyzed the EN1 mRNA and protein expression in four different tissues: epididymal white adipose tissue (WAT), brown adipose tissue (BAT), brain tissue (BR), and skeletal muscles (MUS) from C57BL/6J male mice at 8 weeks of age

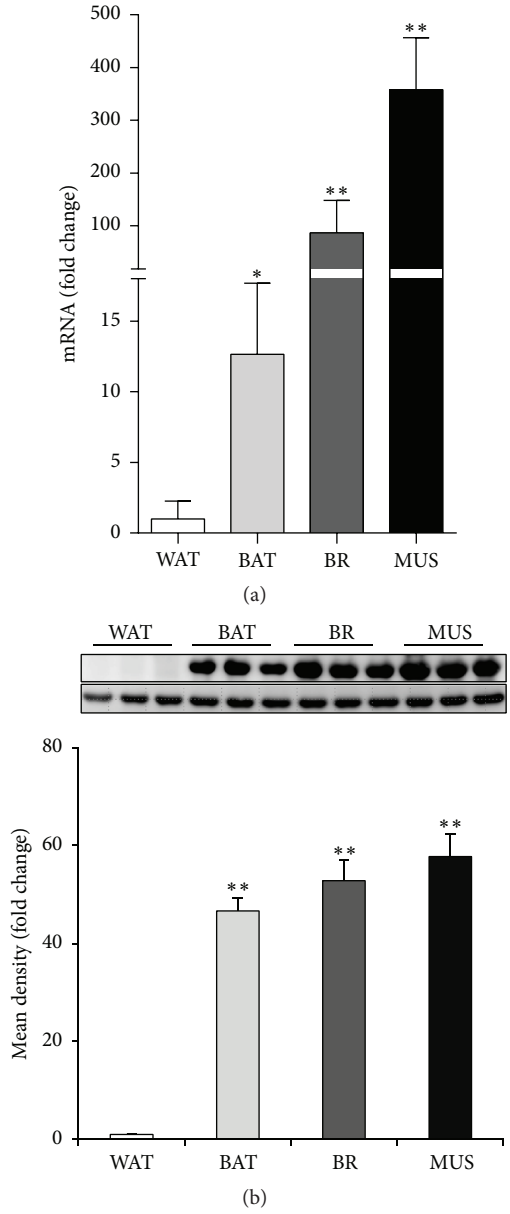

Figure 1: Higher expression of EN1 in BAT than in WAT. (a) The mRNA expression and (b) protein expression of EN1 in WAT, BAT, BR, and MUS were analyzed. Relative levels of EN1 protein were calculated based on densitometry analysis (bottom panel of (b)). Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$–5.

fed with normal chow diet. The result showed that both the EN1 mRNA and protein expression were highly enriched in BAT compared with WAT (Figures 1(a)-1(b)). Surprisingly, the EN1 expression in skeletal muscle is several-hundred-fold higher than WAT.

3.2. Expression of EN1 in BAT at Pathophysiological Conditions. Cold exposure is believed to be a most attractive physiological way to activate BAT [5, 21]. Moreover, previous reports have indicated dysfunction of BAT in obesity mice model [3, 4]. To determine the expression of EN1 at pathophysiological conditions, the BAT tissues were analyzed in mice with different treatment, such as room temperature (RT) versus cold exposure (Cold); low-fat diet (LFD) versus high-fat diet (HFD); wild type (WT) versus Ob/Ob mice. Interestingly, the mRNA and protein expression of EN1 were downregulated upon cold exposure (Figures 2(a)-2(b)). In contrast, they were upregulated in obesity mice compared with control mice (Figures 2(c)–2(f)). These results indicate that EN1 might influence BAT lipid contents.

3.3. EN1 Expression during Brown Adipogenesis. The above results led us to hypothesize that EN1 might be involved in brown adipogenesis. To this end, primary brown adipocytes (SCA1+/CD31−/CD11b−; referred to as classical brown adipose tissue progenitor cells) were isolated from fetal C57BL/6J mouse BAT according to previous publications [22, 23] (Figures 3(a)-3(b)). In order to explore the potential role of EN1, we first investigated the mRNA and protein expression of EN1 during brown adipogenesis. Interestingly, the expression of EN1 was downregulated at day 1 and progressively increased during brown adipogenesis up to day 7 (Figures 3(c)-3(d)). These results highlight that EN1 might be involved in brown adipogenesis.

3.4. Overexpression of EN1 Stimulates Brown Adipogenesis. To investigate the possibility of whether EN1 is involved in brown adipogenesis, a lentivirus EN1 overexpression plasmid or empty vector was transfected into primary brown adipocyte and then underwent brown adipogenesis. EN1 expression was successfully upregulated more than 30-fold at three days after viral transduction (Figures 4(a)-4(b)). Interestingly, the lipid accumulation which was assessed by Oil-Red O staining was significantly increased by EN1 overexpression (Figure 4(c)), suggesting that EN1 stimulates brown adipogenesis. Therefore, we investigated the expression of adipogenic genes (AP2, PPARγ2, and C/EBPs), brown adipocyte specific thermogenic proteins, UCP1 and PGC1α, and mitochondrial oxidative phosphorylation (OXPHOS) protein at the end of the brown adipogenesis. Our results showed that both mRNA and protein expression of AP2 and PPARγ2 were dramatically upregulated after EN1 overexpression (Figures 4(d) and 4(f)). In addition, the mRNA expression of C/EBPα was also significantly increased after EN1 overexpression (Figure 4(d)). Furthermore, EN1 overexpression led to significant induction of thermogenic proteins, such as UCP1 and PGC1α, as well as mitochondrial OXPHOS proteins, including ATP5α, UQCRC2, SDHB, and NDUFB8 (Figures 4(e)-4(f)). These results indicated that EN1 promotes brown adipogenesis by upregulating the key adipogenic gene expression.

3.5. Knockdown of EN1 Suppresses Brown Adipogenesis. In order to further verify our results, knockdown of EN1, the complementary approach to overexpression, was applied to brown adipogenesis. Lentivirus encoding Sh-EN1 plasmid or empty vector was transinfected into primary brown adipocytes and underwent brown adipogenesis. We confirmed that EN1 expression was successfully downregulated

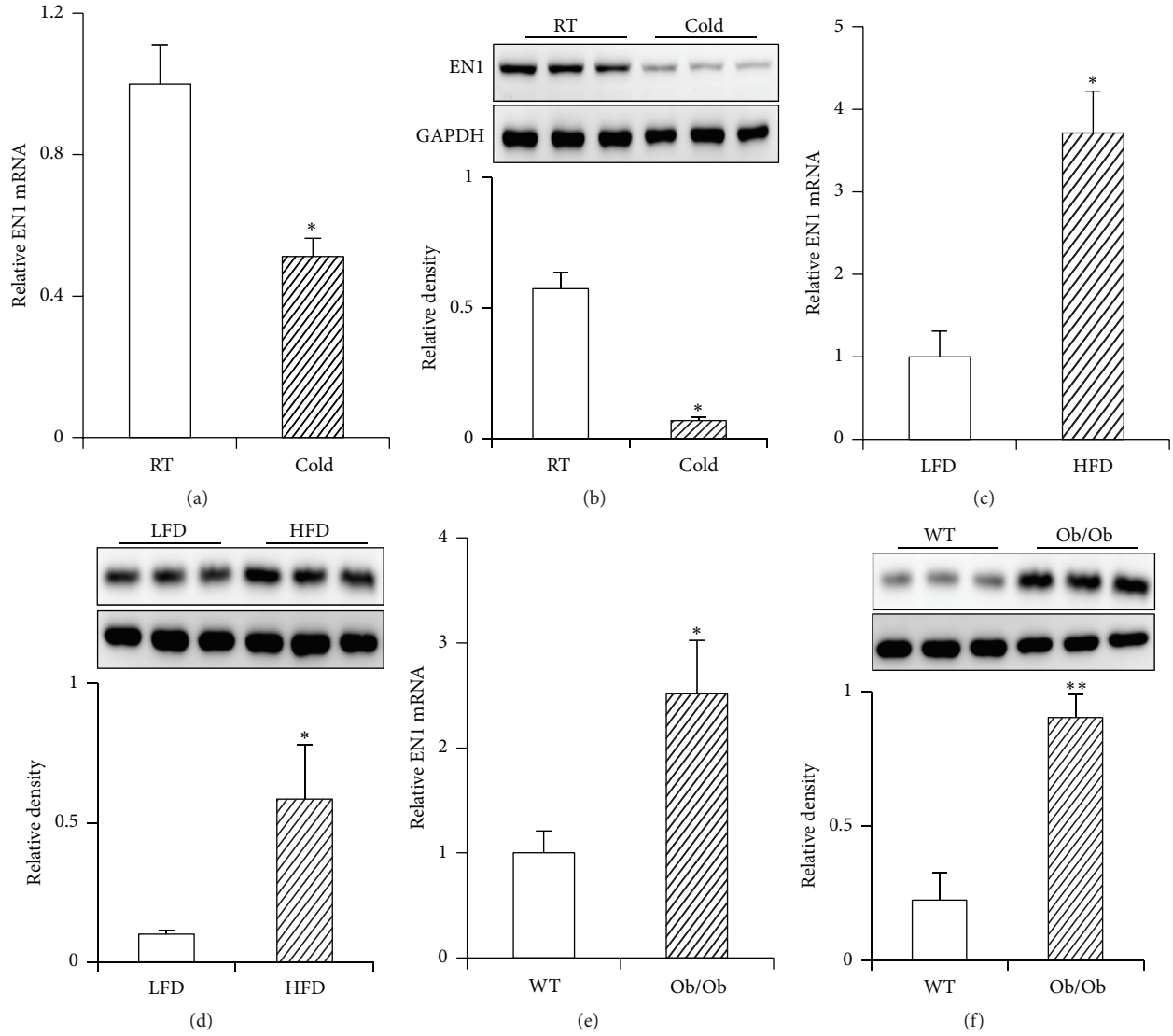

Figure 2: Expressions of EN1 in BAT at pathophysiological conditions. (a, c, and e) mRNA expression and (b, d, and f) protein expressions of EN1 in BAT of mice in different pathophysiological conditions: room temperature (RT) and 4°C for 8 hours (cold); 3-week-old male C57BL/6J mice fed low-fat diet (LFD) or high-fat diet (HFD) for 8 weeks; 8-week-old C57BL/6J (WT) and Ob/Ob mice were analyzed. Relative levels of EN1 protein were calculated based on densitometry analysis (bottom panel of (b), (d), and (f)). Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$–5.

about 75% at three days after viral transduction (Figures 5(a)-5(b)). As expected, lipid accumulation was significantly decreased by EN1 knockdown (Figure 5(c)). Consistently, both mRNA and protein expression of AP2 and PPARγ2 were dramatically downregulated after shEN1 plasmid transfection (Figures 5(d) and 5(f)). Furthermore, the mRNA expression of C/EBPα was also significantly decreased by EN1 knockdown (Figure 5(d)). Moreover, EN1 knockdown led to significant reduction of thermogenic proteins, UCP1 and PGC1α, as well as mitochondrial OXPHOS proteins, such as ATP5α, UQCRC2, SDHB, and NDUFB8 (Figures 5(e)-5(f)). Taken together, the results from Figures 4-5 clearly indicated that EN1 promotes brown adipogenesis.

4. Discussion

In the current study, we investigated the role of EN1 in brown adipogenesis. We demonstrated that EN1 is a positive regulatory factor for brown adipogenesis. To our knowledge, this is first study showing that role of EN1 in brown adipogenesis.

Transplantation of BAT can effectively improve the whole body energy metabolism and prevent metabolic disorders, such as obesity and insulin resistance [3, 4, 7]. Increased amount and/or activity of BAT are critical approaches to combat obesity and related diseases. Generating large volume of brown adipocytes by manipulating genes including EN1 and chemicals could be one of the best approaches in the future.

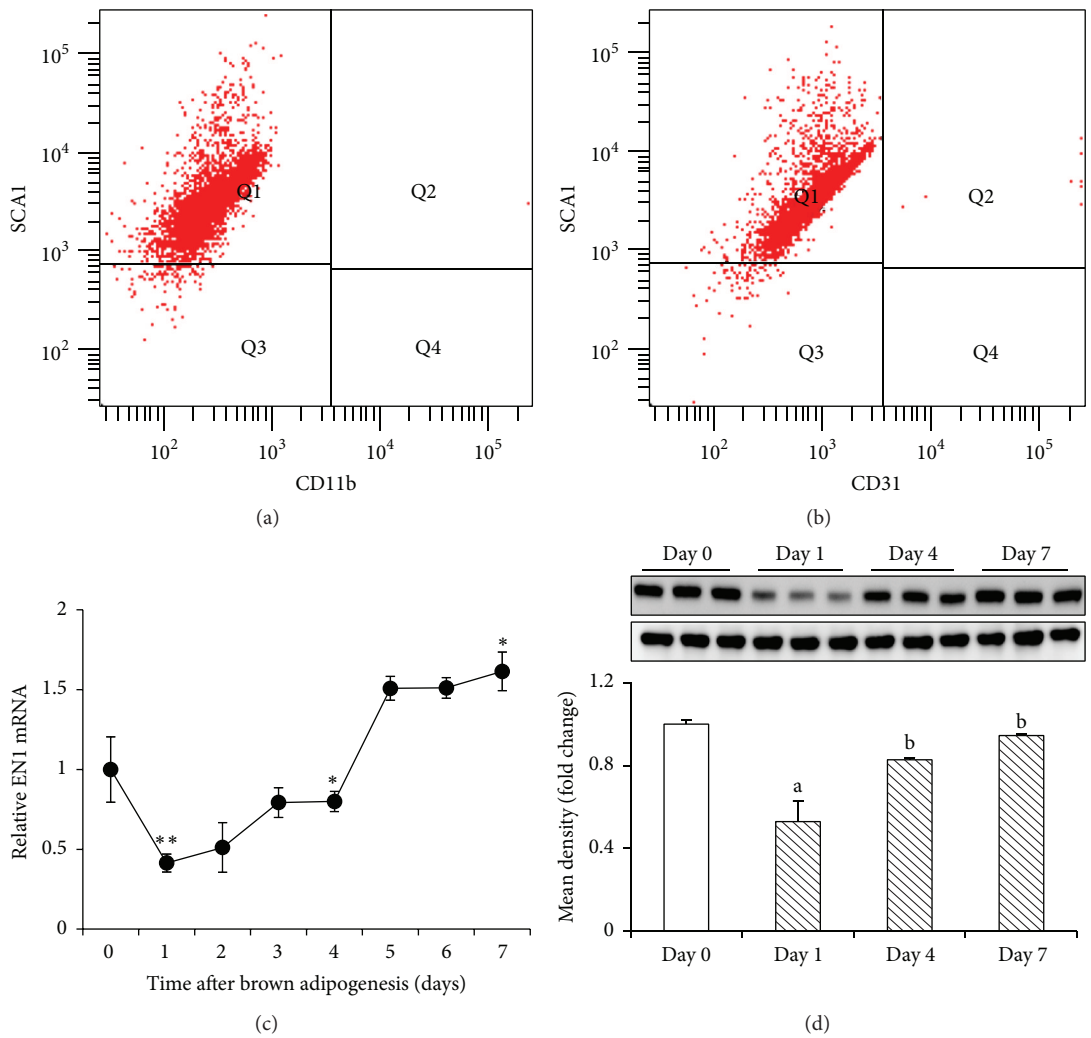

Figure 3: EN1 expression during brown adipogenesis. (a-b) Primary brown adipocyte (SCA1+/CD31−/CD11b−) was isolated from fetal C57BL/6J mouse BAT and (c) the mRNA and (d) protein expression of EN1 were analyzed during brown adipogenesis. Relative levels of EN1 protein were calculated based on densitometry analysis (bottom panel of (d)). Data were analyzed by unpaired Student's t-test in (c). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control or analyzed by one-way ANOVA with Tukey's post hoc test; $n = 3$–5 per group.

EN1 is well known as one of the brown adipocyte lineage-tracing markers [17], yet little is known about its functions in brown adipogenesis. We found that expression of EN1 was higher in BAT than WAT (Figures 1(a)-1(b)). EN1 is required for midbrain and cerebellum development and patterning of the limbs [18, 19]. Consistently, we also confirmed a high expression of EN1 in the brain and muscle.

To investigate the potential role of EN1 in brown adipogenesis, we first determined the expression of EN1 during brown adipogenesis. Interestingly, expression of EN1 is downregulated at day 1 and progressively increased during brown adipogenesis upon day 7. These results prompt us to hypothesize that EN1 might be involved in brown adipocyte differentiation. Interestingly, we found that overexpression of EN1 accelerated while knockdown of EN1 suppressed the brown adipocyte differentiation (Figures 4-5). These results imply that EN1 positively regulates brown adipogenesis.

The major transcriptional factors such as PPARγ2 and C/EBPα interacted with each other to commit adipocyte differentiation [24]. PPARγ1 and PPARγ2, two isoforms of PPARγ, are essential transcriptional factors for both white and brown adipogenesis [25–28]. However, mutation of PPARγ receptor in mice results in impairments of brown adipocyte thermogenic function and recruitment in BAT [29]. In our hands, overexpression of EN1 upregulates while knockdown of EN1 downregulates the PPARγ2 and C/EBPα expression (Figures 4-5). These results indicate that EN1 is involved in brown adipogenesis via regulating expression of PPARγ2 and C/EBPα. However, the exact underlying molecular regulatory mechanisms are remained to be studied.

PGC1α, a coactivator of PPARγ2, also plays an important role in adaptive thermogenesis in BAT by regulating mitochondrial biogenesis and upregulates the expression of UCP1

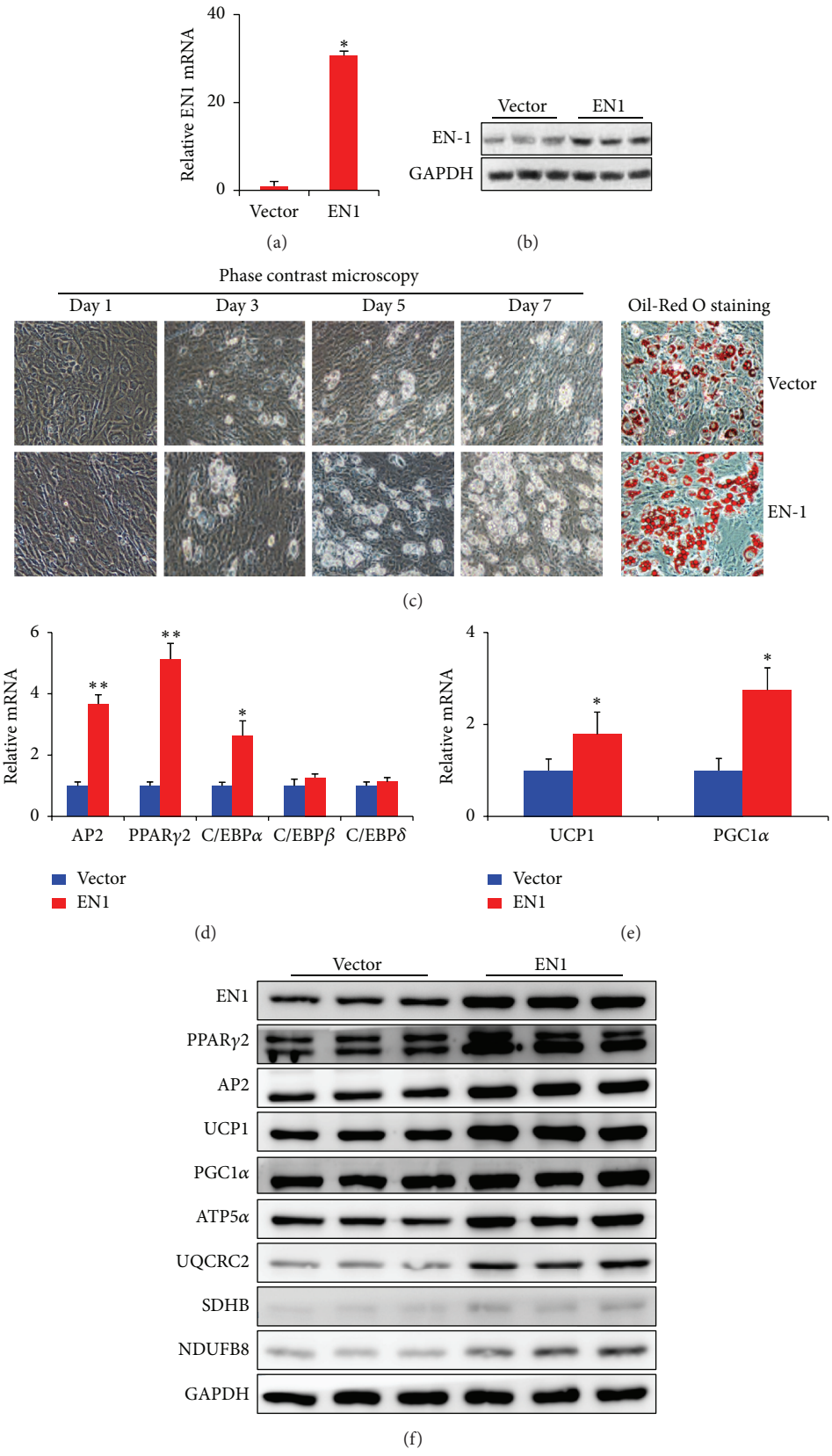

FIGURE 4: Overexpression of EN1 stimulates brown adipogenesis. Lentiviral mediated overexpression of EN1 was confirmed by (a) RT-PCR or (b) western blot analysis. Brown adipogenesis was assessed by (c) phase contrast microscopy and Oil-Red O staining. (d, e) Adipogenic and thermogenic gene expression and (f) BAT related protein expressions were analyzed during brown adipogenesis. Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$.

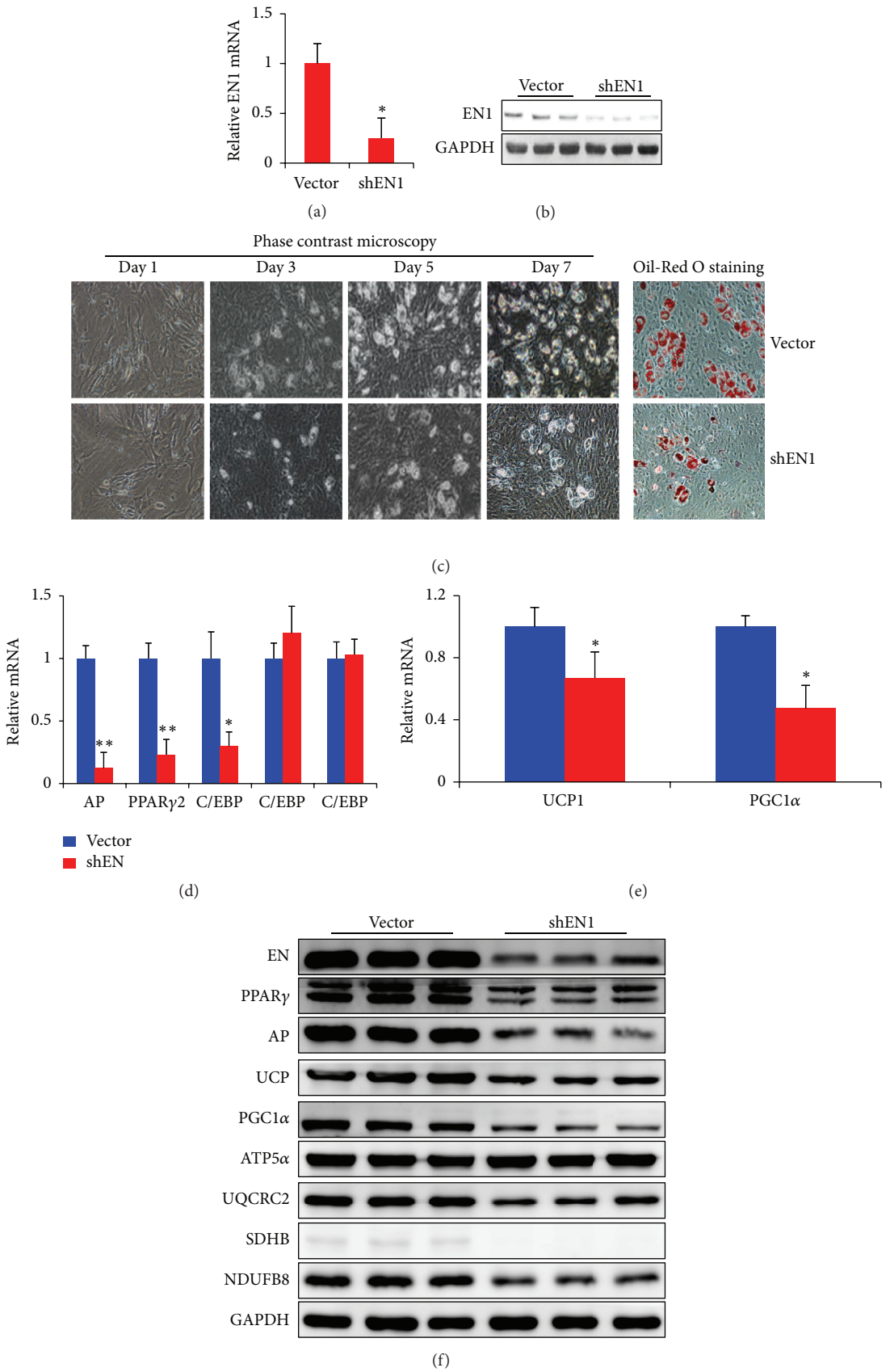

Figure 5: Knockdown of EN1 suppresses brown adipogenesis. Lentiviral mediated knockdown of EN1 expression was confirmed by (a) RT-PCR or (b) western blot analysis. Brown adipogenesis was assessed by (c) phase contrast microscopy and Oil-Red O staining. (d, e) Adipogenic and thermogenic gene expression and (f) BAT related protein expressions were analyzed during brown adipogenesis. Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$.

[8, 30–33]. Furthermore, PRDM16 and C/EBPβ complex synergistically enhances the activity of PGC1α [11, 32]. Interesting to us, the degree of EN1 expression has significant effect on BAT function as determined by mitochondrial OXPHOS protein expression and BAT specific marker gene expression including UCP1 and PGC1α (Figures 4-5).

In addition, the expression of EN1 is dramatically downregulated upon cold exposure, while it is increased in BAT of obesity mice. Moreover, it was significantly increased in BAT of both the high-fat diet (HFD) and Ob/Ob mice, which showed diminished functions of BAT [34, 35]. It was well known that the brown adipogenesis often associated with BAT function [36]. We therefore emphasize that EN1 might reflect the size of lipid droplets in BAT, since it is well known that HFD cause BAT hyperplasia with enlarged lipid droplet. On the other hand, cold exposure results in smaller lipid droplet size in BAT. In line with this speculation, EN1 also might be regulated by beta 3 adrenergic signaling which is important for cold induced BAT activation. Further studies are needed to clarify these hypotheses.

In conclusion, our current study demonstrated that EN1 positively regulated brown adipogenesis and BAT functions via increasing the expressions of thermogenic proteins as well as mitochondrial OXPHOS proteins.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Chuanhai Zhang and Yibing Weng have contributed equally to this work.

Acknowledgments

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Research Article

Association between Toll-Like Receptor 4 and Occurrence of Type 2 Diabetes Mellitus Susceptible to Pulmonary Tuberculosis in Northeast China

Yuze Li,[1,2] Dianzhong Li,[2] Jinfeng Zhang,[1] Shurui Liu,[1] Haijun Chen,[3] and Kun Wu[1]

[1]*Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150081, China*
[2]*Department of the Fourth Internal Medicine, The Fourth Hospital of Heilongjiang Province, Harbin 150500, China*
[3]*CT Department, Heilongjiang Province Hospital, Harbin 150036, China*

Correspondence should be addressed to Kun Wu; wukun_15000@126.com

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The purpose of this study is to explore why type 2 diabetes mellitus (T2DM) patients are susceptible to pulmonary tuberculosis through detection of serum Toll-like receptor 4 (TLR_4), an important immune-related receptor, especially in terms of content and TLR_4 gene polymorphism. Patients with T2DM complicated by pulmonary tuberculosis (T2DMTB) were selected as the case group and T2DM patients without tuberculosis were selected as the control group. Forty patients in each group were randomly selected and their serum TLR_4 levels were detected and compared. Determination of six sites of TLR_4 gene polymorphism was carried out in 238 T2DMTB patients and 310 patients with T2DM, and results showed that the serum TLR_4 content of the T2DMTB group was significantly lower than that of the T2DM group ($p < 0.05$). The six sites of TLR_4 gene polymorphism did not show significant associations with T2DMTB risk. No statistically significant differences in genotype distributions were observed between T2DMTB patients and patients with T2DM when studied using the recessive and dominant genetic models. How two diseases with contradictory nutritional statuses can occur in the same person is difficult to explain from environmental factors perspective alone. Future research should study the causes of T2DMTB from the perspective of genetics.

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) complicated by tuberculosis (TB) (T2DMTB) is rapidly increasing, resulting in detrimental effects on the economy and human health.

Clinical symptoms reveal that environmental factors play important roles in T2DM and TB. Because T2DMTB may be attributed to multiple causes, clinical experience indicates the traditional perspective that the two diseases are related in terms of nutrition. TB is associated with malnutrition, while T2DM is associated with excess nutrients. Thus, environmental factors, among which nutrition is one of the most important, alone, cannot explain the pathogenesis of two seemingly conflicting diseases occurring simultaneously in the same individual. In this case, genetic factors may be responsible for the development of T2DMTB [1–3], but we are not the first who are aware of host genetic factors that are important to determine the risk of type 2 diabetes mellitus complicated by tuberculosis. García-Elorriaga et al. [4] analyzed the association of inflammatory cytokine polymorphisms and T2DMTB in Mexico.

Toll-like receptor 4 (TLR_4), a pattern recognition receptor, can recognize pathogen-associated molecular patterns (PAMPs) [5] and exacerbate and release inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFN-γ [6]. *Mycobacterium tuberculosis* presents liposaccharides (LPS), a type of PAMP. When TLR_4 recognizes the LPS of *M. tuberculosis*, inflammatory cytokines are induced and released by the pathway of TLR_4 [7–9]. This pathway is an important element in the innate immune system of the human body. TLR_4, as an important mediator of inflammation, can recognize a variety of pathogens, including Gram-negative and Gram-positive bacteria [10]. Previous studies have shown that human TLR_4

is a potentially important gene that may affect the onset of T2DM [11–13]. The receptor may be related to susceptibility to some infections that can induce diseases such as TBs [14–18]. To date, no study has yet reported the association between TLR_4 gene polymorphisms and the risk of T2DMTB in the Chinese population.

We believe that TLR_4 gene polymorphisms are a good starting point for studying T2DMTB. To be able to establish preventive measures for high-risk people as far as possible, in this study, we investigate the relationship between TLR_4 gene polymorphisms and T2DMTB risk in Northeast China.

2. Material and Methods

2.1. Study Subject and Sample Collection. We recruited 238 cases with T2DMTB as the case group and 310 cases with T2DM as the control group. T2DMTB patient information was obtained from the Fourth Medical Ward, Heilongjiang Province Tuberculosis Control Center, from September 2013 to June 2015. T2DM patient information was obtained from the Second Department of Outpatient Services, Heilongjiang Province Tuberculosis Control Center, from September 2013 to November 2014.

Subjects with a history of blood-transmitted diseases, such as AIDS, hepatitis B, hepatitis C, or other endocrine diseases, were excluded from this study. Blood samples were taken from the patients, and DNA was obtained and stored in 193 K. Both doctors and study subjects provided consent to participate in this work, and the Ethics Committee of Harbin Medical University approved of this research.

2.2. Immunohistochemical Method. To determine whether serum TLR_4 levels were consistent with the gene expression level, 40 serum samples were collected from the T2DMTB group and T2DM group, respectively, according to the principle of random. Serum TLR_4 contents were detected by using an immunohistochemical method following the manufacturer's instructions (Beijing Cheng Lin Biological Technology Co., Ltd.).

2.3. Tag SNP Selection and Genotyping. Tag SNPs of TLR_4 gene were evaluated and selected by using the HapMap database (https://hapmap.ncbi.nlm.nih.gov/) with the following criterion: a minor allele frequency (MAF) > 0.05 in the Chinese Han population in the National Center of Biotechnology Information Database; linkage disequilibrium (LD) blocks were established by using Chinese LD maps and $r^2 > 0.8$. Six tag SNPs representing the genetic information of TLR_4 were selected for genotyping: rs1927914 located in the promoter region; rs11536879, rs1927911, and rs1927907 located in the intron region; and rs11536889 and rs7873784 located in the $3'$-UTR region.

According to the manufacturer's instructions, genomic DNA was extracted from peripheral blood using a Tiangen DNA Blood Mini kit (Tiangen Biotech Co., Ltd., Beijing, China). All SNPs were genotyped using fluorogenic $5'$-nuclease assay (TaqMan SNP Genotyping Assay, Applied Biosystems, Foster City, CA, USA). For quality control, 20% of all of the samples were performed in delicate form randomly for each SNP. The concordance rate of these repeated samples was 100%.

2.4. Statistical Analysis. Numerical data are expressed as mean ± SD. Student's t-test was performed to analyze differences between the T2DMTB and T2DM groups.

Fisher's exact test was used to evaluate the Hardy-Weinberg equilibrium (HWE) in the subjects. The chi-square test or Fisher's exact test was used to identify statistical differences in the distributions of clinic pathological characteristics. Univariate and multivariate unconditional logistic regression were used to estimate crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs), all of which measured the associations between the risk factors of T2DMTB compared with T2DM. Two-sided $p < 0.05$ was considered as statistically significant. Statistical analysis was carried out by using SAS software (version 9.1.3, SAS Institute, Cary, NC).

3. Results

3.1. Demographic Characteristics. A total of 548 people participated in this study, including 238 cases of T2MDTB and 310 cases of T2MD. Among the patients, 338 were male and 210 were female. The mean ages of T2DMTB and T2DM patients were 51.97 ± 11.87 years and 58.73 ± 11.46 years, respectively. The demographic characteristics of the patients are shown in Table 1.

3.2. Serum TLR_4 Levels of Patients with T2DMTB and Patients with T2DM. The serum TLR_4 levels of subjects with T2DMTB were significantly lower than those of subjects suffering from T2DM ($p < 0.0001$). No differences in the basic characteristics of the participants, including gender ($p = 0.260$) and age ($p = 0.631$), were observed (Table 2).

3.3. Distributions of SNP Genotypes between Patients with T2DMTB and Patients with T2DM. Genotype distributions did not show statistically significant deviations from HWE for all SNPs in this study (Table 3).

3.4. Distribution of Allelic Genes of SNPs between Patients with T2DMTB and Patients with T2DM. The distributions of allelic genes of six SNPs of the TLR_4 gene were not statistically significant between patients with T2DMTB and patients with T2DM. Details of these distributions are summarized in Table 4.

3.5. SNPs and T2DMTB Risk. The SNPs of TLR_4 were not significantly associated with T2DMTB risk. No significant differences in genotype distributions between patients with T2DMTB and patients with T2DM were observed using the recessive and dominant genetic models (Table 5). However, potential trends may provide useful information for future studies. In rs7873784, compared with the GG genotype, the

Table 1: Demographic characteristics of the subjects.

Variable	T2DMTB	T2DM	Total	p value
Age				
⩽50	104	68	172	
50–60	73	108	181	<0.0001
60–70	45	80	125	
⩾70	16	54	70	
Gender				
Male	167	171	338	0.0003
Female	71	139	210	
BMI				
⩽18.5	35	8	43	
18.5–24	112	64	176	<0.0001
⩾24	91	238	329	
Smoking				
Yes	132	86	218	<0.0001
No	106	224	330	
Drinking				
Yes	108	94	202	0.0003
No	130	216	346	
Glucose				
<5.0	12	13	25	
5.0–7.2	48	88	136	0.0857
>7.2	178	209	387	
Insulin use				
Use	112	196	308	
No use	126	112	238	<0.0001
Unclear	0	2	2	
Hypoglycemic drug use				
Use	45	224	269	
No use	193	80	273	<0.0001
Unclear	0	6	6	

Table 2: Serum TLR_4 levels of patients with T2DMTB and T2DM.

Variable	T2DMTB	T2DM	p value
Gender (male/female)	40 (20/20)	40 (25/15)	0.260
Age (years)	51.17 ± 6.70	51.85 ± 6.20	0.631
TLR_4 (ng/mL)	15.27 ± 2.52	19.40 ± 1.80	<0.0001

GC genotype presented lower risks of T2DMTB ($OR_{adjusted}$ = 0.69, 95% CI: 0.42–1.14, p = 0.15). In rs11536879, AG genotype carriers showed decreased risk of T2DMTB compared with the GG genotype ($OR_{adjusted}$ = 0.68, 95% CI: 0.43–1.08, p = 0.10).

4. Discussion

A previous study showed that patients with T2DM demonstrated four to eight times increased risk of tuberculosis compared to patients without T2DM. For instance, TB in T2DM patients was 5 times more prevalent than in non-T2DM patients in some regions in the USA, 5.4 times more prevalent than in non-T2DM patients in Australia, and 6.8 times more prevalent than in non-T2DM patients in Mexico [4].

In the clinic, we discovered that T2DMTB patients present uneven changes in inflammatory cytokines such as TNF-α and IFN-γ. Clinical examination of simple TB yielded a positive IFN-γ test, but the results of nearly all patients with T2DMTB were negative. TLR_4 is an important element of the endogenic immune system and it can induce and release inflammatory cytokines [5]. TLR_4 is an important element of the innate immune system of the human body. Thus, we hypothesize that serum TLR_4 exhibits obvious changes in T2DMTB patients. We examined TLR_4 serum levels in patients with T2DMTB and patients T2DM to determine whether TLR_4 is involved in the susceptibility of T2DM patients suffering from TB in Northeast China. While no study has yet proven that TLR_4 polymorphisms are related to T2DMTB or T2DM, other studies [19–22] indicate that TLR_4 polymorphisms present statistically significant differences between healthy people and patients with TB.

Our study results are different from those of Wu et al. [23], especially in terms of the frequencies of the GG genotype of SNP rs7873784 in TLR_4 (OR = 2.136; 95% CI: 1.312–3.478) and the CC genotype of rs3764879 in TLR_8 (OR = 1.982; 95% CI: 1.292–3.042). It was also significantly higher in the TB group than in the healthy group. Arji et al. [24] found TLR_4 interactions influencing protection against TB in Moroccan patients. The present work and that of Jahantigh et al. [25] both demonstrated no significant relation between TLR_4 and TLR_9 polymorphisms and TB. Sánchez et al. [26] reported that they did not find any association between TLR_4 polymorphic variants. These findings suggest that the gene polymorphisms were not involved in any risk factor for pulmonary TB in the Colombian population [26]. The same result was obtained by Xue et al. [27], who discovered that these polymorphisms were rare in the Southeastern Chinese population and not linked to susceptibility to TB. Newport et al. [28] also have studied that, but result was that no association between TLR_4 Asp299Gly and TB was observed.

The result of our study showed that it was negative. The reasons behind this result are first the size of the sample which was not enough. The second one is the geographic regions and genetic factors, because we were focused on the people of Northeast China in our work just only.

The purpose of this study is to determine why patients with T2DM easily develop pulmonary TB. Thus, we did not detect TLR_4 gene polymorphisms in healthy cases and we did not compare changes in TLR_4 gene polymorphisms among healthy cases, patients with T2DM, and patients with T2DMTB. Changes in TLR_4 gene polymorphisms may have already occurred in most patients with T2DM. In this case, a false negative result may appear if a comparison was carried out between T2DM patients and patients suffering from T2DMTB only. Such a phenomenon would also confirm some reports that T2DM is characterized by chronic inflammation.

Some studies have recently mentioned that the risk genotypes of rs1927914 are significantly linked with diabetic foot ulcers [29]; the research team of Singh [30] also found that the combined genotype risks of TLR_4 SNPs rs10759931

Table 3: Distributions of SNP genotypes and Hardy-Weinberg equilibrium.

	T2DMTB No. (%)	T2DM No. (%)	p value	Hardy-Weinberg χ^2	Hardy-Weinberg p value
rs7873784					
GG	243 (86.17)	236 (83.69)			
GC	35 (12.41)	45 (15.96)	0.19	0.56	0.46
CC	4 (1.42)	1 (0.35)			
rs11536889					
GG	176 (61.97)	181 (64.41)			
GC	93 (32.75)	86 (30.61)	0.83	0.81	0.37
CC	15 (5.28)	14 (4.98)			
rs1927914					
TT	90 (31.91)	102 (36.17)			
CT	145 (51.42)	140 (49.65)	0.50	0.53	0.47
CC	47 (16.67)	40 (14.18)			
rs1927911					
TT	97 (34.28)	96 (34.16)			
CT	139 (49.12)	148 (52.67)	0.48	2.92	0.09
CC	47 (16.61)	37 (13.17)			
rs1927907					
GG	145 (51.24)	147 (52.31)			
AG	123 (43.46)	119 (42.35)	0.96	2.12	0.15
AA	15 (5.30)	15 (5.34)			
rs11536879					
AA	230 (82.44)	215 (77.62)			
AG	41 (14.70)	56 (20.22)	0.20	1.04	0.31
GG	8 (2.87)	6 (2.17)			

Table 4: Distributions of allelic genes of SNPs between patients with T2DMTB and patients with T2DM.

SNP	Allelic genes	T2DMTB		T2DM		p value
		No.	%	No.	%	
rs7873784	G allele	521	92.36	517	91.67	0.74
	C allele	43	7.64	47	8.33	
rs11536889	G allele	445	78.35	448	79.72	0.61
	C allele	123	21.65	114	20.28	
rs1927914	T allele	325	57.62	344	60.99	0.43
	C allele	229	42.38	220	39.01	
rs1927911	T allele	333	58.83	340	60.50	0.59
	C allele	233	41.17	222	39.50	
rs1927907	G allele	413	72.97	413	73.49	0.89
	A allele	153	27.03	149	26.51	
rs11536879	A allele	501	89.78	486	87.73	0.30
	G allele	57	10.22	68	12.27	

(odds ratio [OR] 1.50, $p = 0.05$) and rs1927914 (OR 1.48, $p = 0.05$) were significantly linked to retinopathy in T2DM. These works support the idea that T2DM is a chronic inflammatory disease. It is possible that T2DM patients already had TLR_4 gene polymorphism. Therefore, we considered these points as the third reason.

Two groups of patients were included in our study. For each group, we concerned about the changing of TLR_4 gene polymorphism and no changing of TLR_4 levels in the blood. The reason of that was hidden behind the changing in the TLR_4 gene, because serum TLR_4 is a sensitivity index but not a specific index. Chronic inflammation could directly lead to

Table 5: Associations between the SNPs of TLR_4 and DMTB risk.

Genotype	Crude OR (95% CI)	p value	Adjusted OR (95% CI)*	p value
rs7873784				
GG	1.00		1.00	
GC	0.76 (0.47–1.22)	0.25	0.69 (0.42–1.14)	0.15
CC	3.89 (0.43–35.01)	0.23	3.16 (0.33–30.74)	0.32
CC/(GG + GC)	4.04 (0.45–36.41)	0.21	3.34 (0.34–32.38)	0.30
(GC + CC)/GG	0.82 (0.52–1.31)	0.41	0.75 (0.46–1.22)	0.25
rs11536889				
GG	1.00		1.00	
GC	1.12 (0.78–1.60)	0.54	1.04 (0.71–1.51)	0.85
CC	1.11 (0.52–2.36)	0.79	1.08 (0.49–2.39)	0.85
CC/(GG + GC)	1.07 (0.51–2.26)	0.86	1.07 (0.49–2.34)	0.87
(GC + CC)/GG	1.12 (0.79–1.57)	0.53	1.04 (0.73–1.49)	0.82
rs1927914				
TT	1.00		1.00	
CT	1.17 (0.81–1.69)	0.39	1.25 (0.85–1.84)	0.25
CC	1.33 (0.80–2.21)	0.27	1.35 (0.80–2.29)	0.27
CC/(TT + CT)	1.21 (0.77–1.91)	0.42	1.18 (0.73–1.91)	0.49
(CT + CC)/TT	1.21 (0.85–1.71)	0.29	1.28 (0.89–1.84)	0.19
rs1927911				
TT	1.00		1.00	
CT	0.93 (0.65–1.34)	0.70	0.93 (0.63–1.36)	0.71
CC	1.26 (0.75–2.10)	0.38	1.22 (0.72–2.09)	0.46
CC/(TT + CT)	1.31 (0.82–2.09)	0.25	1.28 (0.79–2.08)	0.36
(CT + CC)/TT	1.00 (0.70–1.41)	0.98	0.99 (0.69–1.43)	0.96
rs1927907				
GG	1.00		1.00	
AG	1.05 (0.75–1.47)	0.79	1.03 (0.72–1.48)	0.86
AA	1.01 (0.48–2.15)	0.97	0.95 (0.44–2.05)	0.89
AA/(GG + AG)	0.99 (0.48–2.07)	0.98	0.93 (0.44–1.99)	0.85
(AG + AA)/GG	1.04 (0.75–1.45)	0.80	1.02 (0.72–1.44)	0.90
rs11536879				
AA	1.00		1.00	
AG	0.68 (0.44–1.07)	0.09	0.68 (0.43–1.08)	0.10
GG	1.25 (0.43–3.65)	0.69	1.35 (0.45–4.04)	0.59
GG/(AA + AG)	1.33 (0.46–3.89)	0.60	1.44 (0.48–4.31)	0.51
(AG + GG)/AA	0.74 (0.49–1.12)	0.16	0.75 (0.48–1.15)	0.19

Note. *Age, gender, BMI, smoking, drinking, insulin use, and hypoglycemic drug use were adjusted.

changes in TLR_4 in the blood or other gene polymorphisms that could lead to TLR_4 changes in the blood. Our study was limited by considering just TLR_4 gene polymorphisms. Other inflammatory gene polymorphisms that promote development of T2DMTB may exist.

The results of this study reveal that TLR_4 changes at the molecular level are insignificant because the changes at the gene level may be not significant. Our results also demonstrated no significant link between the six SNPs of TLR_4 studied in this work and the susceptibility of patients with T2DMTB in Northeast China. Future studies may be performed to determine the causes of T2DMTB from the perspective of genetics. We aim to determine the marker gene polymorphism in T2DM and show how it can be complicated by TB.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Control of Cross Talk between Angiogenesis and Inflammation by Mesenchymal Stem Cells for the Treatment of Ocular Surface Diseases

Fei Li and Shao-zhen Zhao

Tianjin Medical University Eye Hospital, The College of Optometry, Tianjin 300384, China

Correspondence should be addressed to Shao-zhen Zhao; zhaosz1997@sina.com

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Angiogenesis is beneficial in the treatment of ischemic heart disease and peripheral artery disease. However, it facilitates inflammatory cell filtration and inflammation cascade that disrupt the immune and angiogenesis privilege of the avascular cornea, resulting in ocular surface diseases and even vision loss. Although great progress has been achieved, healing of severe ocular surface injury and immunosuppression of corneal transplantation are the most difficult and challenging step in the treatment of ocular surface disorders. Mesenchymal stem cells (MSCs), derived from various adult tissues, are able to differentiate into different cell types such as endothelial cells and fat cells. Although it is still under debate whether MSCs could give rise to functional corneal cells, recent results from different study groups showed that MSCs could improve corneal disease recovery through suppression of inflammation and modulation of immune cells. Thus, MSCs could become a promising tool for ocular surface disorders. In this review, we discussed how angiogenesis and inflammation are orchestrated in the pathogenesis of ocular surface disease. We overviewed and updated the knowledge of MSCs and then summarized the therapeutic potential of MSCs via control of angiogenesis, inflammation, and immune response in the treatment of ocular surface disease.

1. Introduction

Cornea is the transparent front part of the eye. It is composed of epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. Limbal stem cells (LSCs) are residing at the basal layer of the limbus and could differentiate into terminal epithelium cells for replacement. In the stage of corneal damage, LSCs could generate epithelial cells for repair [1]. As damage progresses, angiogenesis and lymphangiogenesis in the avascular cornea result in the infiltration of neutrophils and macrophages as well as Th1 cells for further attack. As the pathological process involves regions of corneal limbus, LSCs are lost and dysfunctional and fail to replace the damaged epithelial cells, leading to blindness [2]. In this case, LSCs and corneal transplantation are the most feasible option to improve ocular surface damage and vision. Although the success rate of transplantation is high, graft rejection still occurs resulting from preoperative high-risk factors, postoperative inflammation, angiogenesis, lymphangiogenesis, and immune response [3–5]. To date, it has been reported that more than 10 million patients have been suffering from corneal blindness in the world [6].

Mesenchymal stem cells (MSCs) are originated from multiple adult tissues such as bone marrow, liver, and adipose tissue. As pluripotent cells, MSCs could differentiate into different cell types [7]. Besides their differentiation potential, MSCs exert immunomodulatory and anti-inflammation effects on the surrounding cells by the release of secreted cytokines [8]. When cocultured with LSCs, MSCs could stimulate LSCs proliferation and growth factor expression in vitro [9]. Therefore, MSCs therapy could be a promising approach for ocular surfaces diseases via control of lymphangiogenesis, inflammation, and immune response. In the review, we will first overview the knowledge of MSCs and then focus on how MSCs control the pathological cross talk between lymphangiogenesis and inflammation in the treatment of corneal diseases.

2. Characteristic and Potential of MSCs

2.1. Definition of MSCs. MSCs have been isolated from several adult tissues, including bone marrow, adipose tissue, liver, dental pulp, endometrium, muscle, amniotic fluid, placenta, and umbilical cord blood [10–12]. MSCs have pluripotent or multipotent properties as well as a great potential of differentiating into mesodermal cell lineages (e.g., adipocytes, osteocytes, and chondrocytes) and nonmesodermal cell lineages (e.g., cardiomyocytes, hepatocytes-like cells, neurons, astrocytes, and endothelial cells) both in vivo and in vitro. In addition, it is found that pericytes present in several organs, such as skeletal muscle and pancreas, also express the very same markers used by MSCs [13]. They could share many of the differentiation characteristics of MSCs in vitro [14]. Thus, the perivascular niche can be regarded as a subset of MSCs [13–16]. Due to the lack of specific markers for these cells, the authentic MSCs are difficult to identify. To resolve this problem, the International Society for Cellular Therapy has provided the minimum criteria for defining multipotent MSCs: plastic adherent under standard culture conditions; positive for the expression of CD105, CD73, and CD90 surface markers; absent for the expression of CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR surface markers; and capable of differentiating into osteocytes, adipocytes, and chondrocytes under a specific stimulus in vitro [17].

2.2. Differentiation Ability. MSCs have both endothelial and epithelial tissue coding genes and could be promoted to differentiate into endothelial- or epithelial-like cells both in vitro and in vivo [18–20]. Under specific conditions, MSCs could differentiate into corneal epithelial cells, keratocyte-like cells, and endothelial-like cells to repair damaged corneas [21–25]. However, some lines of evidence found that the replaced cells derived from MSCs do not behave as true tissue cells [26]. This might be due to the inconsistent differentiation protocols and heterogeneity of cell population [27, 28].

2.3. Immunomodulatory and Anti-Inflammation Potential. MSCs are potent regulators of immune response and inflammation. MSCs could be activated by the inflammatory microenvironment through exposure to proinflammatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and effector T cells [29, 30]. In vitro, MSCs interact with innate and acquired immune response and inhibit the proliferation and function of T cells, B cells, dendritic cells, and natural killer cells [31–33]. The regulation of immune cells by MSCs mainly comes from a panel of cytokines secreted by MSCs, including IL-6, IL-10, transforming growth factor-β (TGF-β), metal matrix proteinase (MMP), prostaglandin E2 (PGE2), indoleamine-2,3-dioxygenase (IDO), human leukocyte antigen-G5 (HLA-G), and nitric oxide (NO) [34, 35]. Second, MSCs could decrease the expression levels of Th1 cell factors (IFN-γ and IL-2) and increase the expression levels of Th2 cell factors (IL-4 and IL-10), thereby promoting immune response of naïve CD4+ cells toward the Th2-type response [32]. Third, when cocultured with lymphocytes, MSCs produce PGE2 and TGF-β to promote regulatory T-cells (Tregs) differentiation and expansion [36, 37]. It is well known that Tregs have the capacity to suppress the proliferation of activated T cells. Therefore, modulation of Tregs has been suggested as the main mechanism of MSCs in maintaining immune tolerance for allografts survival in organ transplantation [38].

The application of MSCs in organ transplantation has been tested in rat and primate transplantation models. MSCs seemed to significantly suppress immune rejection and prolong graft survival in the heart, liver, kidney, pancreas, and other solid organs [39–42]. Following systemic infusion, MSCs could not only migrate to lesion but also be trapped in lungs and other organs [43]. Although it is not fully clear, MSCs homing is regulated via chemokine, chemokine receptors, intracellular signals, adhesion molecules, and proteases, such as stromal cell-derived factor-1α (SDF-1α) and C-X-C chemokine receptor type 4 (CXCR4) [44–47]. It is currently under investigation whether improved MSCs homing could bring about better therapeutic effect.

However, MSCs therapy is not always successful and even accelerates allograft rejection after organ transplantation [57]. The controversial results might be influenced by many factors including infusion time and dose, administration mode, and homing efficiency of MSCs.

2.4. Angiogenic Property of MSCs. A growing body of evidence has shown the regulation effect of angiogenesis by MSCs. This effect was mainly attributed to the modulation of angiogenic factors produced by MSCs. For instance, vascular epithelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) both promote the migration and proliferation of vascular endothelial cells [58]. In cardiac ischemia repair, MSCs stimulate neovascularization of infarct tissue through upregulating VEGF to improve cardiac function [59]. The effects may be associated with the role of TLR2 [60]. However, the direct interaction between VEGF and TLR2 in MSCs is not clear. In addition, some reports demonstrated that MSCs could be additional important cells for proangiogenesis to form provisional granulation matrix in the proliferation phase of wound healing [61].

Different from the effect of proneovascularization in ischemic tissues and tumors, MSCs showed an opposite effect on inflammation-related corneal angiogenesis after chemical injury. This action was associated with the significantly smaller mean neovascularized area and a reduced expression level of VEGF [62], which might be attributed to the expression of high level of thrombospondin-1 (TSP-1), which inhibits VEGF [63]. These results suggested that the different microenvironment would modulate different behavior and function of MSCs. Below, we overviewed MSCs applications in ocular surface diseases.

3. MSCs in Ocular Surface Diseases (Table 1)

3.1. Corneal Wound Repair. Corneal injury is caused by thermal injury, alkali or acid burns, and immune or hereditary disorders and leads to corneal inflammation, neovascularization, conjunctivalization, impaired vision, and even

Table 1: Experiments of MSCs in ocular surface diseases.

Diseases	Experimental outcomes	The mechanisms	Factors	References
Chemical injury	Reduce corneal opacity	Reduce inflammation and neovascularization	↑ TSG-6	[48]
Chemical injury	Protect the corneal surface	Reduce inflammation and neovascularization Reduce CD4+ cells infiltration	↑ IL-10, IL-6, TSP-1, and TGF-β1 ↓ IL-2, IFN-γ, and MMP-2	[63]
Alkali burn	Improve wound healing	Enhance the recovery of corneal epithelium Decrease the CNV area	↓ MIP-1α, TNF-α, and VEGF	[62]
Chemical burn	Restructure damaged corneal surface	Inhibit inflammation and angiogenesis	↓ IL-2 and MMP-2	[71]
Chemical burn	Affect profiling of IL-17-secreting cells	Mainly modulate non-Th17 cells and partially suppress Th17 cells	↓ IL-17	[73]
Corneal allotransplantation	Prolong grafts survival	Inhibit immune response Suppress early inflammation Reduce the activation of APCs	↑ TSG-6	[52]
Corneal allotransplantation	Prolong grafts survival	Prevent T-cells response Regulate the balance of Th1/Th2 to Th2 Increase CD4+CD25+Foxp3+ Treg	↑ IL-10 and IL-4 ↓ IL-2 and IFN-γ	[53]
Corneal allotransplantation	Prolong grafts survival	Reduce NK cells infiltration Increase CD4+ Foxp3+ Treg Suppress peripheral immune response Promote an immunoregulatory milieu	↓ IL-6, IL-1β, and IFN-γ	[54]
DES	Protect ocular surface	Reduce the CD4+ T cells		[55]
Dry eye secondary to chronic GVHD	Reduce clinical symptoms and improve dry eye scores	Increase the CD8+CD28− T cells Regulate the balance of Th1/Th2 to Th2		[56]

TSG-6: TNF-α-stimulated gene/protein 6; MMP: metal matrix proteinase; CNV: cornea new vessel; TSP-1: thrombospondin-1; TNF-α: tumor necrosis factor-α; MIP-1α: macrophage inflammatory protein-1α; IFN-γ: interferon-γ; VEGF: vascular epithelial growth factor; DES: dry eye syndrome; GVHD: graft-versus-host disease; APCs: antigen-presenting cells.

blindness [64]. LSC is an essential cell population for corneal epithelium regeneration and ocular surface reconstruction. Unfortunately, LSC loss and dysfunction in corneal limbus trigger severe inflammation and neovascularization [65]. To be noted, LSC transplantation remains as the effective strategy to treat LSCD but it is challenged by limited donors and allograft rejection [66, 67].

Alternative therapy is to improve resident LSC and corneal epithelial cell (CEP) expansion for repair [10, 68, 69]. In vitro studies showed that MSCs could stimulate LSC and CEP proliferation when they were cocultured. Both systemic and topical administration of MSCs have been shown to accelerate corneal regeneration and healing [48, 49]. In practice, to increase local concentration, MSCs were injected into the injured cornea with a hollow plastic tube [70], through subconjunctival administration of MSCs [62] or through transplantation of MSCs with tissue engineering materials, such as amniotic membrane (AM) [50, 71] and nanofiber scaffolds [72]. The strategy of combining with tissue engineering materials is better for cornea recovery than MSCs or tissue engineering materials used alone [51].

Besides stimulation of LSC proliferation, MSCs injection effectively alleviates inflammation and neovascularization in the injured cornea. MSCs increased the expression of the anti-inflammatory cytokines IL-10, TGF-1, TNF-α-stimulated gene/protein 6 (TSG-6), and the antiangiogenic factor thrombospondin-1 (TSP-1) and reduced the expression levels of the proinflammatory factors IL-2, IFN-γ, IL-17, macrophage inflammatory protein-1α (MIP-1α), and MMP-2 [70, 71, 73, 74]. Ultimately, neutrophil and macrophage infiltration is largely reduced.

3.2. Corneal Transplantation. Corneal transplantation is the most common form of human tissue transplantation. Comparing with other types of organ transplantation, normal-risk corneal transplants have an exceptionally high success rate of up to 80% over 5 years, which is mainly based on the specific immune privilege of cornea including low-level expression of MHC I and MHC II, the lack of indigenous antigen-presenting cells (macrophages or Langerhans cells), the absence of lymphoid and blood vessels, and anterior chamber associated immune deviation (ACAID) [75–77]. However, as risk factors, preexisting lymphangiogenesis and blood vessel together with inflammation promote graft injection [78].

Currently, a broad range of treatment strategies have been proposed to increase the duration of grafts survival. The leading method for preventing transplant rejection is corticosteroids and immunosuppression such as cyclosporine A, FK-506 [79–81]. However, long-term immunosuppression could produce drug toxicity and potential complications; the used dosages are limited. More recent works have focused

on endothelia transplantation (only endothelia are diseased) and lamellar transplantation (endothelia are not diseased) to reduce immune rejection [82–84].

MSCs administration has been widely tested in corneal transplantation and inconsistent results have been reported. Some studies demonstrated that pretransplant infusion of MSCs was effective to prolong graft survival; meanwhile, MSCs used postoperatively are less effective, especially for kidney and heart transplantation. This might be explained by the fact that preoperative infusion of MSCs modulated Tregs expansion and induced immune tolerance before occurrence of inflammation and immune progress [85–87]. Oh et al. [52] suggested that pretransplant systemic infusion of human MSCs inhibited immune response largely due to suppressing early inflammation caused by surgery and reducing the activation of antigen-presenting cells (APCs) in both cornea and draining lymph nodes (DLNs). The role of MSCs was primarily exerted by secreting the soluble anti-inflammatory protein TSG-6. However, Jia et al. [53] have shown that MSCs prolonged corneal allograft survival time only when injected immediately after the surgery and preoperative administration exerted no significant effect. The cornea immune privilege triggers delayed-type hypersensitivity which may take longer for the corneal allograft to activate an immune response than other types of solid organ transplantation.

By contrast, Oh et al. [88] were the first who examined the immunomodulatory effects of MSCs in corneal transplantation in a pig-to-rat model. Allogeneic rat MSCs were applied topically to corneal grafts for 2 h immediately after transplantation. They observed the increased expression levels of Th2-type cytokine (IL-10) in the rejected grafts from MSC-treated rats and a shift from Th1 to Th2 cell type following MSCs administration. However, MSCs injection failed to prolong pig corneal xenograft survival in rats. Similarly, Fuentes-Julián et al. [89] obtained an insufficient conclusion about adipose-MSCs treatment to prevent corneal grafts rejection. In the study, local or systemic adipose-MSCs administration in rabbit corneal transplantation models at normal- or high-risk rejection does not prolong the graft survival. The used adipose-MSCs lacked immunomodulatory ability on T lymphocytes and immunophenotypical secretion molecules, which may be the reason why the adipose-MSCs destroyed the innate ocular immune privilege and accelerated rejection [89].

All of the above observations demonstrated that MSCs would be a potential therapeutic tool for corneal allograft transplantation and the molecular mechanisms of action need to be further studied. Importantly, many factors including the source of MSCs, the infusion time, the dose of injection, and the mode of administration would influence the best results of MSCs treatment on corneal transplantation. In addition, MSCs in combination with immunomodulatory drugs are an alternative treatment. Intravenous transfusion of MSCs and cyclosporine A (CsA) achieved a synergistic effect on suppressing immune rejection of corneal grafts [53].

3.3. Dry Eye Syndrome. Dry eye syndrome (DES) or keratoconjunctivitis sicca (KCS) is the major ocular surface disease affecting ranges from 7% to 33% of the worldwide population [90, 91]. Dry eye is a common ocular complication associated with chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation, occurring in 60% of patients [92, 93]. DES is characterized by deficiency of the tear film components (lipid, aqueous, and mucin). Although the mechanisms of DES are yet unknown, inflammation in the lacrimal gland and the ocular surface plays a key role in the pathogenesis of the disorder. DES is also an autoimmune disease with immune-mediated destruction in the whole process [94, 95]. Immunohistochemistry of lacrimal gland shows immune cell infiltration and loss of acinar epithelial cells, and the expression of proinflammatory cytokines is increased [96]. Studies have suggested that the proinflammatory factors inhibit neurotransmitter release resulting in insufficient secretion of lacrimal gland [97]. In addition, the lacrimal gland could be involved as a target in several systemic and autoimmune diseases including Sjögren syndrome, sarcoidosis, and chronic GVHD [96]. The current treatment strategies, including tear replacement, anti-inflammatory drugs, and punctual occlusion, often fail to resolve the underlying problems of DES.

Topical application of MSCs could be a safe and available treatment for periocular diseases with immune involvement, such as KCS/DES [98, 99]. Allogeneic adipose-MSCs were implanted around lacrimal glands in dogs with KCS [100]. The implanted cells effectively reduced clinical signs during a 9-month follow-up. Lee et al. [55] also demonstrated that periorbital administration of MSCs could protect the ocular surface in a murine model of DES. In the intraorbital gland and ocular surface, the CD4+ T-cells infiltration was reduced. MSCs suppressed inflammation and increased aqueous tear production [55]. In a recent clinical practice, 22 patients with refractory dry eye secondary to chronic GVHD were treated with MSCs, 12 of whom showed reduced symptoms with improved dry eye scores. The results were accompanied by increasing the number of CD8+CD28−T cells, which suggest that MSCs regulate the balance between Th1 and Th2 [56]. There is a human Phase I/II clinical trial involving allogeneic MSCs treatment for primary Sjögren syndrome (http://clinicaltrials.gov/ct2/show/NCT00953485). Based on the double effect between inflammation and immunomodulation, MSCs are a promising source to treat dye eye syndrome.

4. Summary and Perspectives

The ocular immune and angiogenic privileges act as a barrier to protect corneal function. Ingrowth of new blood vessels orchestrates inflammatory cell infiltration leading to inflammation and impaired epithelial cell repair. MSCs have shown therapeutic effect in corneal surface diseases by several lines of mechanisms: inhibition of inflammatory cell infiltration and inflammatory cytokine release, modulation of the switch from Th1 cell type toward Th2 cell type, activation of Treg cells, and stimulation of epithelial cell regeneration (Figure 1). Therefore, MSCs would be a very promising tool in the treatment of corneal diseases. Further studies would focus on increase of MSCs homing efficiency, time, and safety

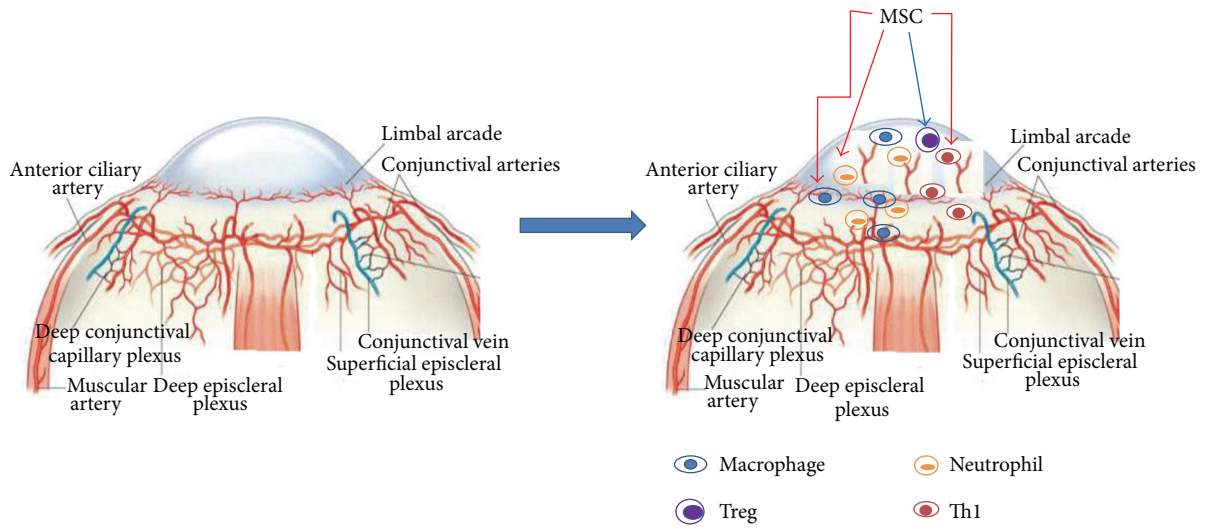

Figure 1: Modulation of immune cells and inflammatory cells by MSCs in corneal surface diseases. Cornea is the avascular and transparent front part of the eye, maintained by immune and angiogenesis privilege. In the occurrence of injury and transplantation, ingrowth of blood and lymph vessels into the cornea leads to infiltration of inflammatory cells and Th1 cells, which strengthen the inflammation and damage the cornea structure. MSCs have several protective functions by (1) inhibition of the inflammatory cell infiltration and inflammatory cytokine release, (2) activation of Treg cells for immune tolerance, (3) tuning the transition from Th1 cells toward Th2 cells, and (4) improving epithelium regeneration (not shown).

and MSCs administration, together with evaluation of MSC-based therapies when dealing with ocular surface diseases.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Transcatheter Arterial Infusion of Autologous CD133$^+$ Cells for Diabetic Peripheral Artery Disease

Xiaoping Zhang,[1,2] Weishuai Lian,[1] Wensheng Lou,[3] Shilong Han,[1] Chenhui Lu,[1] Keqiang Zuo,[1] Haobo Su,[3] Jichong Xu,[1] Chuanwu Cao,[1] Tao Tang,[1] Zhongzhi Jia,[1] Tao Jin,[1] Georges Uzan,[4] Jianping Gu,[3] and Maoquan Li[1,2]

[1]*Department of Interventional and Vascular Surgery, Shanghai Tenth People's Hospital, Tongji University, No. 301 Middle Yan Chang Road, Shanghai 200072, China*
[2]*Institution of Interventional and Vascular Surgery, Tongji University, No. 301 Middle Yan Chang Road, Shanghai 200072, China*
[3]*Department of Interventional Radiology, Nanjing First Hospital, No. 68 Changle Road, Nanjing, Jiangsu 210001, China*
[4]*Unité de Recherche INSERM 602, 94807 Villejuif Cedex, France*

Correspondence should be addressed to Jianping Gu; jrxglmq@163.com and Maoquan Li; cjr.limaoquan@vip.163.com

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Microvascular lesion in diabetic peripheral arterial disease (PAD) still cannot be resolved by current surgical and interventional technique. Endothelial cells have the therapeutic potential to cure microvascular lesion. To evaluate the efficacy and immune-regulatory impact of intra-arterial infusion of autologous CD133$^+$ cells, we recruited 53 patients with diabetic PAD (27 of CD133$^+$ group and 26 of control group). CD133$^+$ cells enriched from patients' PB-MNCs were reinfused intra-arterially. The ulcer healing followed up till 18 months was 100% (3/3) in CD133$^+$ group and 60% (3/5) in control group. The amputation rate was 0 (0/27) in CD133$^+$ group and 11.54% (3/26) in control group. Compared with the control group, $TcPO_2$ and ABI showed obvious improvement at 18 months and significant increasing VEGF and decreasing IL-6 level in the CD133$^+$ group within 4 weeks. A reducing trend of proangiogenesis and anti-inflammatory regulation function at 4 weeks after the cells infusion was also found. These results indicated that autologous CD133$^+$ cell treatment can effectively improve the perfusion of morbid limb and exert proangiogenesis and anti-inflammatory immune-regulatory impacts by paracrine on tissue microenvironment. The CD133$^+$ progenitor cell therapy may be repeated at a fixed interval according to cell life span and immune-regulatory function.

1. Backgrounds

Angiopathy and neuropathy are commonly recognized as the basic pathological changes of diabetes, leading to the development of foot complications in diabetic patients [1]. The treatment of purely diabetic neuropathy includes neuronutrition treatment, restricting weight bearing, and foot care therapy, but with limited effects [2]. Ischemia caused by angiopathy is another important factor preventing healing. One of the main features of the angiopathy in patients with diabetic peripheral artery disease (PAD) is that it usually involves both great and small blood vessels.

PAD in diabetics is often multisegmental, typically infrapopliteal, and poorly compensated [3]. With the advancement of surgical skill and the merging of newly developed endovascular instruments, a large number of the aortic-iliac, femoral-popliteal, and infrapopliteal artery lesions can be treated to reestablish sufficient blood supply of the lower limb at macrovascular level; however, there are 20–30% of patients not considered candidates, resulting in amputation as the only option [4, 5]. Moreover, the microvascular lesion in diabetic PAD still cannot be resolved by the current surgical and interventional technique [6].

Endothelial progenitor cells (EPCs) have the capacity of differentiation to mature toward endothelial cells, angiogenesis, and repairmen of impaired vascular walls [7]; therefore, we assumed that EPCs have the therapeutic potential to cure the microvascular lesion of diabetic PAD [8]. Available

evidence suggests that local administration of selected and unselected autologous bone marrow derived cells represents a safe and effective means of inducing therapeutic angiogenesis in patients with critical limb ischemia, though the reported benefits were variable [9–13]. Previous studies have demonstrated that cells that express the $CD133^+$ surface marker have been characterized as immature progenitor cells with high proliferative, vasculogenic, and regenerative capacity *in vitro* and *in vivo* [14–16]. Although the majority of the population of $CD133^+$ positive cells are $CD34^+$ positive, they constitute a population of progenitor cells able to differentiate into mature endothelial cells [15]. Importantly, peripheral $CD133^+$ positive progenitor cells contain a subpopulation of $CD34^-/133^+$ cells, which are functionally more potent than $CD34^+/CD133^+$ cells [14]. We hypothesized that that autologous $CD133^+$ stem cell transplantation may induce vasculogenesis, improve the perfusion of morbid limb, and restore ambulatory function in patients with PAD.

In this study, after successful endovascular infra-aorta revascularization of the macroblood vessels, we enriched $CD133^+$ cells from peripheral blood derived mononuclear cells (MNCs) and reinfuse the cells intra-arterially through a catheter to improve the microvascular angiogenesis, evaluating their efficacy and immune-regulatory impact on diabetic subjects with PAD.

2. Methods

2.1. Study Design. The study was a prospective, nonrandomized trial. All patients assigned to $CD133^+$ cells treatment group ($CD133^+$ group) and some patients assigned to control group were enrolled in the Tenth People's Hospital of Tongji University (Shanghai, China) and the ethic committee of Tenth People's Hospital of Tongji University approved the protocol. Some patients assigned to the control group were enrolled in the Nanjing First Hospital (Jiangsu, China). All patients provided written informed consent. All patients enrolled were assigned to $CD133^+$ cells treatment group ($CD133^+$ group) and control group of their own volition. This study is to evaluate the efficacy and immune-regulatory impact of intra-arterial infusion of autologous $CD133^+$ cells on diabetic subjects with PAD.

Clinical Trial Registration. This trial is registered with URL: http://www.clinicaltrials.gov/. Unique identifier: NCT02474381.

2.2. Patient Enrollment and Grouping. Diabetic PAD patients aged ≥18 years with Rutherford categories 2 to 5 were included to assess the eligibility for this study. All patients, who agreed to participate in the study, could voluntarily choose whether to or not to receive autologous $CD133^+$ progenitor cell treatment.

In this study, $CD133^+$ cells were used to stimulate angiogenesis and reconstruct efficient microvascular blood supply; therefore, similar hemodynamic status in main branch was essential to meet homogeneity in both groups before patient entry. The candidates, who failed in intraluminal revascularization of infra-aorta (iliac and femoral-popliteal) and 1 infrapopliteal (anterior/posterior tibial, fibular) arteries of the affected limb, would be excluded from the study.

Other exclusion criteria were as follows: ① hemoglobin < 10 mg/dL, ② creatinine clearance < 30 mL/min, ③ previous history of stem/progenitor cell therapy, ④ paralysis because of central neural system disease, ⑤ accidental amputation or bone fracture of target limb because of trauma after entry, ⑥ stop of antiplatelet medication after entry, ⑦ smoking or resmoking after entry, and ⑧ malignant tumor.

2.3. Treatment of Infra-Aorta and Infrapopliteal Artery Lesion. Computed tomographic angiography (CTA) was performed to firstly analyze the condition of vascular lesion and then digital subtraction angiography (DSA) was performed to precisely identify the lesions of infra-aorta and the infrapopliteal arteries before treatment.

The treatment of infra-aorta artery lesion was restrictedly performed with intraluminal technique (balloon dilation and/or stent implantation) despite the grade of the lesion according to TASC II classification. The arterial sheath was introduced into the contralateral femoral artery, and then the revascularization of the target limb was accomplished by antegrade approach.

After the previous procedure, the lesions of the infrapopliteal arteries were reevaluated by DSA. By means of balloon dilation, at least one of the anterior/posterior tibial and fibular arteries achieved an obvious direct blood supply to the foot.

The goal of the above procedures is to completely restore the normal main trunk hemodynamic status of the target limb.

2.4. Autologous $CD133^+$ Cells Collection and Preparation. After successful revascularization of infra-aorta and infrapopliteal procedure, 100 mL peripheral blood was collected through the femoral artery sheath in patients and sent to East China Stem Cell Bank (Zhongyuan Union Cell & Gene Engineering Corporation Ltd. (Zhongyuan Union), Tianjin, China) for $CD133^+$ cells sorting and enrichment. Mononuclear cells are separated from the whole blood by density gradient centrifugation with Ficoll separating medium (GE Healthcare (Amersham Biosciences), Uppsala, Sweden), and then $CD133^+$ cells are selected using magnetic-activated cell sorting with CliniMACS cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The selected cells were mixed with 50 mL sodium chloride injection, which contains human albumin and heparin sodium in the blood bag, and then sent back to hospital stored in 4°C. All collection and preparing procedures were finished within 6 hours.

The selected cells also need to take a quality test; otherwise the cells would be discarded and the patient would be excluded from the study. Selected $CD133^+$ cells were tested for viability, purity, and sterility. The quality standards are as follows: cell number $\geq 1 \times 10^7$, no visible precipitate in cell suspension, $CD133^+$ purity ≥ 97% $CD133^+$ based on flow cytometry, viable cell ≥ 90%, and endotoxin ≤ 2 EU/mL.

2.5. Cell Infusion Procedure. A catheter was introduced into the popliteal artery of the target limb at tibial plateau level. The $CD133^+$ cells suspension was drawn into a 50 mL syringe and infused through the catheter by an injection pump timing to 30 minutes.

For the control group, 50 mL cell-free sodium chloride injection containing human albumin and heparin sodium was infused through the catheter as placebo.

2.6. Medication and Life Style Change. Both groups were asked to receive continuous medication for the diabetes, hyperlipidemia, and hypertension under the advice of specialized physicians. Antiplatelet treatment with 100 mg of enteric-coated aspirin and 75 mg clopidogrel daily, as well as statins administration for stabilizing of the arterial plaque, was also demanded.

Besides these medications, all candidates were restrictedly asked to quit smoking after entry.

2.7. Follow-Up and Endpoints. The patients were followed up at 18 months.

The primary endpoints were defined as the aggravation of ulcer (developing new or larger or deeper ulcers) and the amputation (above metatarsal level). The ulcer healing and amputation status were observed monthly.

The change of Rutherford classification, $TcPO_2$ of dorsum pedis, and ABI were recorded to evaluate the blood perfusion of the limb at 6 and 18 months as the second endpoints.

As proven, the stem cells promote angiogenesis through stimulation of endothelial cell proliferation, migration, and survival by paracrine of high levels of vascular endothelial growth factor (VEGF) [17]. In addition to the regenerative properties, stem cells have an immune-regulatory capacity and induce immunosuppressive effects in a series of situations [18]. Human stem cells have been found to suppress Interleukin-6 (IL-6) expression in activated macrophages, which plays a key role in inflammatory response in wound healing [14]. Thus, the serum concentrations of VEGF and IL-6 before and at 1, 2, and 4 weeks after the $CD133^+$ cells infusion were tested to evaluate the proangiogenesis and immunoregulatory impact of the procedure and its duration.

2.8. Statistical Analysis. Baseline characteristics were summarized. Independent t-test was used to test for differences between the groups for continuous variables and Pearson Chi-Square test was used for categorical variables. Adverse events were summarized. Pearson Chi-Square test was used to test for differences between groups in percent of subjects with amputations. Cox regression tests were used to test for differences in the distributions of amputation-free time. Changes of blood perfusion status, proangiogenesis (VEGF), and immunoregulatory (IL-6) factor in groups are presented descriptively and calculated with Independent t-test.

3. Results

3.1. Baseline Characteristics of Patients. As the flow diagram (Figure 1) shows, totally 60 subjects were included initially. 30 patients agreed to receive autologous $CD133^+$ cells treatment and other 30 patients agreed to participate as control. In $CD133^+$ group, 2 patients with TASC II type D lesion failed in the femoral-popliteal revascularization and 1 patient failed in the infrapopliteal revascularization. In control group, 1 patient with TASC II type C lesion and 1 patient with TASC II type D lesion failed in the femoral-popliteal revascularization, and 2 patients failed in the infrapopliteal revascularization. Therefore 53 patients (27 for $CD133^+$ group and 26 for control group) were enrolled in the study finally. The baseline characteristics of these patients were described in Table 1. Except for hyperlipidemia, there was no variance of baseline status between the two groups.

3.2. Quality of Selected $CD133^+$ Cells. From 27 blood samples of $CD133^+$ group, the total number of selected cell was 10.29 ± 3.30 ($\times 10^7$, range 5.20–15.20), and the viable cell accounted for $96.88 \pm 1.03\%$ (range 95.00–98.70%). No patient showed adverse side effect (skin itching, asthma, blood pressure decrease, and unconsciousness) during their autologous $CD133^+$ cells infusion procedure.

3.3. Ulcer Healing. Among 53 patients, 8 cases (3 in $CD133^+$ group and 5 in control group) were affected with skin ulcer and toe gangrene. In $CD133^+$ group, one case had the ulcer on the left heel and one had it on the right big toe, which healed within 1 month; one case had dry gangrene on the right 3 toes (1st, 2nd, and 4th) and healed after 3 months by dropping toes and daily debridement. In control group, 3 cases had toe ulcer healed within 1 month, and 1 case suffered from a new ulcer and 1 case kept unhealed within 18 months. The ulcer healing was 100% (3/3) in $CD133^+$ group and 60% (3/5) in control group. There was no significant difference of heal rate between two groups (Pearson Chi-Square, $P = 0.206$).

3.4. Amputation. There was no amputation in $CD133^+$ group. However, 3 cases in the control group had amputation within 18 months, among which one suffered from new ulceration and being worsening afterwards and got amputation at 5 months, the other one with unhealed ulcer had the amputation at 1 month after enrollment, and the third one had amputation at 2 months, who had never got skin ulcer but suffered from rest pain constantly. The amputation rate was 0 (0/27) in $CD133^+$ group and 11.54% (3/26) in control group. There was a trend of fewer and later amputation in $CD133^+$ group, but without significant difference in terms of amputation rate (Pearson Chi-Square, $P = 0.069$) or amputation-free time (COX regression, OR 1.125, 95% CI 0.657–1.928, $P = 0.668$) between the two groups.

3.5. Rutherford Classification, $TcPO_2$ of Dorsum Pedis, and ABI. The blood perfusion condition of the target limb was assessed by the evaluation of Rutherford classification (Table 2) and the test for $TcPO_2$ of dorsum pedis and ABI (Table 3). There was no significant difference observed at 6 months after the procedure; however, the Rutherford classification, $TcPO_2$, and ABI showed obvious improvement at 18 months in $CD133^+$ group than in control group, which indicated a delayed and persistent perfusion-improving benefit of $CD133^+$ cell treatment (Figure 2).

Table 1: Baseline characteristics of patients.

		$CD133^+$	Control	P (t-test)
Age		71.26 ± 9.12	71.62 ± 9.11	0.887
Gender	Male	12	15	0.901†
	Female	12	14	
Type of DM	Type 1	6	9	0.317†
	Type 2	21	17	
Hypertension	+	19	21	0.379†
	−	8	5	
Hyperlipidemia	+	12	4	0.021†
	−	15	22	
Smoking	+	11	10	0.865†
	−	16	16	
CAD	+	11	7	0.288†
	−	16	19	
Hemoglobin, g/L		120.07 ± 16.77	115.77 ± 15.59	0.338
HbA1c, %		8.26 ± 2.08	8.28 ± 2.07	0.954
Serum creatinine, mL/min		80.60 ± 28.73	79.02 ± 22.44	0.824
Total cholesterol, mmol/L		4.55 ± 1.05	4.58 ± 1.02	0.920
Total triglyceride, mmol/L		2.09 ± 2.36	2.47 ± 2.37	0.560
EMG	+	21	20	0.941†
Sensorimotor Polyneuropathy	−	6	6	
TASC II	A	0	2	0.440†
	B	11	8	
	C	8	7	
	D	5	3	
No aorta-iliac and femoral-popliteal artery lesions		3	6	
Rutherford	0	0	0	0.320†
	1	0	1	
	2	12	5	
	3	8	10	
	4	4	5	
	5	3	5	

DM, diabetes mellitus; CAD, coronary artery disease; EMG, electrical test of the muscles; TASC II, Transatlantic Intersociety Consensus II.
Value were represented by mean ± SD, t-test (†Pearson Chi-Square).

3.6. Serum VEGF and IL-6 Concentration. Serum VEGF and IL-6 level in both groups had no difference before the procedure, whereas $CD133^+$ group showed significant increasing of VEGF and decreasing IL-6 level compared to the control since the first week (Table 4). There were also trends that the immune-regulatory effect of $CD133^+$ cells began to return to basic level at 4 weeks after injected into the patients' limb.

4. Discussion

Endothelial cells have the therapeutic potential cure microvascular lesion of diabetic PAD. Many clinical trials have investigated the safety and efficacy of all kinds of EPCs for the treatment of PAD, using cell surface markers-based selected bone marrow or peripheral blood derived MNCs [19]. The most commonly used cell markers is CD34; nevertheless no single marker or combination of markers identifies a pure endothelial progenitor cell population. Researchers have revealed that, with the nature of endothelial cells, CD133 expression is downregulated, whereas CD34 expression is upregulated, and $CD34^-/133^+$ cells subpopulation, a precursor of "classical" $CD34^+/133^+$ EPC, is functionally more potent with respect to homing and angiogenesis [20, 21]. Therefore, we used enriched $CD133^+$ cell to initiate microvascular angiogenesis in diabetic PAD patients. Flow cytometry analysis shows that the majority of cells obtained after $CD133^+$ positive selection are $CD34^+$ positive, the percentage of $CD133^+/34^+$ cells ≥ 98%. This nonrandomized control study firstly reported in the world that $CD133^+$ cell provided the proangiogenesis and immune-regulatory effects on diabetic PAD patients.

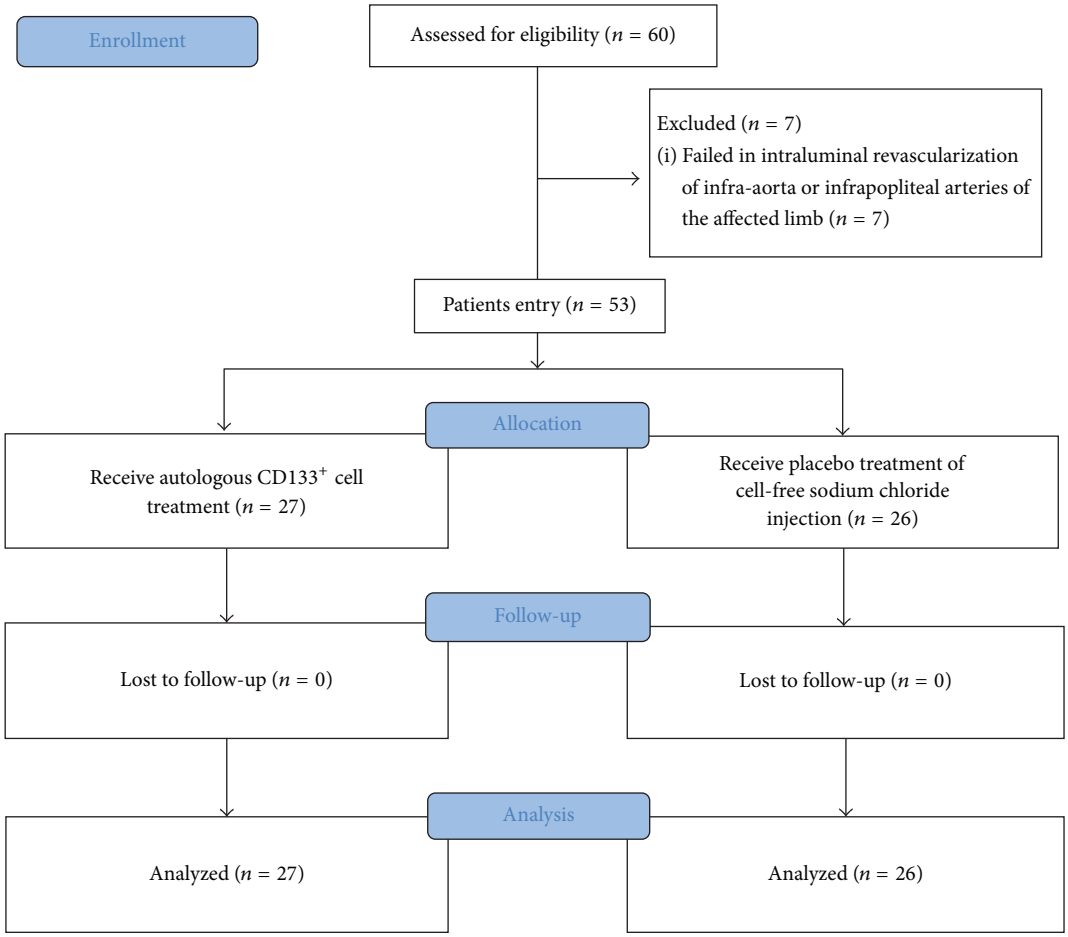

Figure 1: Flow diagram; suggested representation of the flow of participants in a series of diabetic PAD trials.

Table 2: Rutherford classification after treatment.

Case number		$CD133^+$ case	Control	P
6 months	0	1	1	0.114
	1	1	6	
	2	22	13	
	3	3	1	
	4	0	2	
	5	0	1	
18 months	0	1	0	0.004
	1	13	7	
	2	13	6	
	3	0	7	
	4	0	3	
	5	0	0	

Pearson Chi-Square test.

Table 3: ABI and $TcPO_2$ before and after treatment.

		$CD133^+$	Control	P
ABI	Before treatment	0.73 ± 0.13	0.70 ± 0.14	0.417
	6 m	0.81 ± 0.08	0.82 ± 0.12	0.733
	18 m	0.86 ± 0.07	0.81 ± 0.09	0.046
$TcPO_2$ (mmHg)	Before treatment	48.67 ± 9.14	47.38 ± 9.07	0.610
	6 m	56.37 ± 2.76	54.88 ± 6.75	0.305
	18 m	57.41 ± 2.74	54.35 ± 4.80	0.011

ABI: ankle brachial index; m: month.

The results of this study showed the trend of reducing amputation rate, prolonging amputation-free time, and improving ulcer healing of diabetic PAD patients in $CD133^+$ group compared to control group. The perfusion status is significantly improved at 18 months in $CD133^+$ group, while no significant difference has been found at 6 months, comparing ABI and $TcPO_2$ of the affected limb.

The results are consistent with other randomized controlled pilot studies of progenitor cell therapy for critical limb ischemia (CLI). Losordo et al. reported that the autologous $CD34^+$ cells slightly reduced the amputation rate than in control group at 6 months ($P = 0.137$) and 12 months ($P = 0.058$) [10]. Fadini et al. analyzed 37 trials of autologous cell therapy for PAD, finding a significant benefit in terms of limb salvage as compared with placebo treatment and the trend

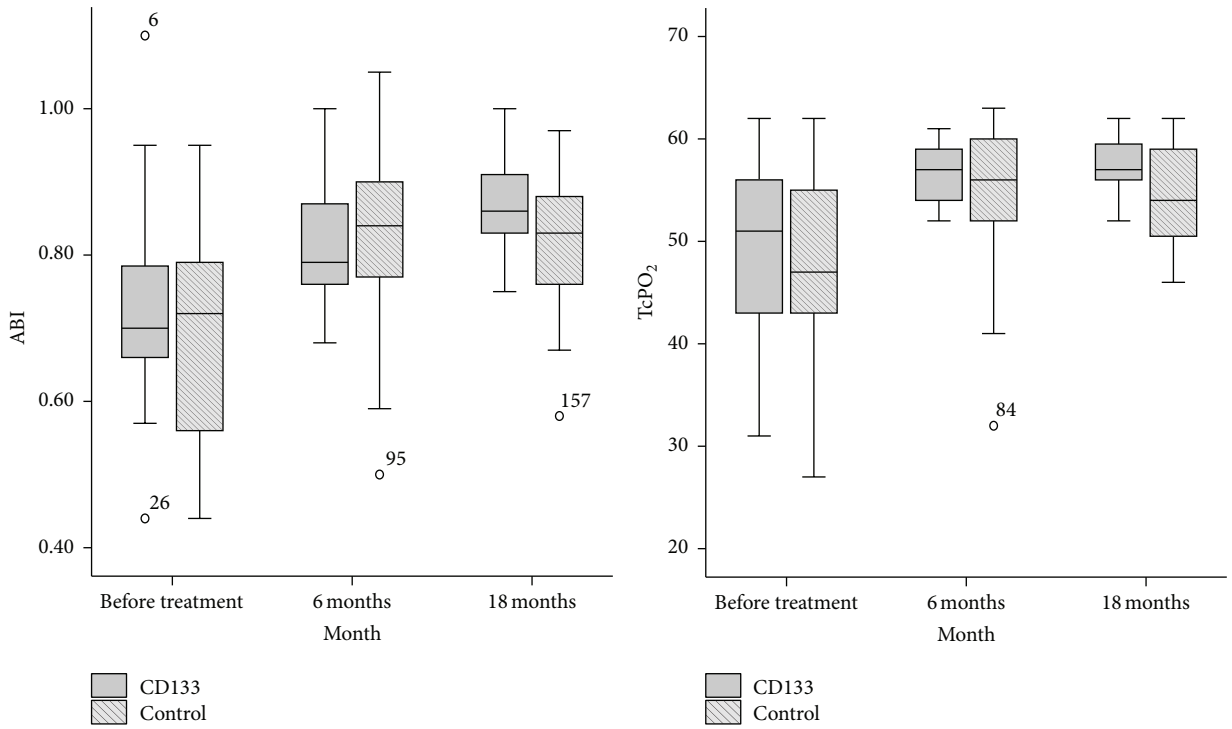

Figure 2: ABI and $TcPO_2$ difference before and after treatment. $TcPO_2$ and ABI were improved significantly in $CD133^+$ group than control at 18 months and did not improve at 1 month, indicating a delayed and persistent perfusion-improving benefit of $CD133^+$ progenitor cell treatment. "∘" represents mild outliers in boxplot, and the small numbers beside it represent the case numbers.

Table 4: VEGF and IL-6 level before and after treatment.

		$CD133^+$	Control	P
VEGF (pg/mL)	Before treatment	69.65 ± 4.43	71.68 ± 5.99	0.166
	1 w	79.01 ± 7.75	74.65 ± 6.74	0.034
	2 w	82.45 ± 4.74	74.07 ± 8.00	0.000
	4 w	78.61 ± 5.04	72.88 ± 6.64	0.001
IL-6 (pg/mL)	Before treatment	124.53 ± 19.94	114.24 ± 25.79	0.109
	1 w	98.66 ± 17.85	111.16 ± 21.60	0.026
	2 w	71.00 ± 25.19	96.25 ± 16.98	0.000
	4 w	81.22 ± 20.22	101.16 ± 22.27	0.001

w: week.

toward improving ABI and $TcPO_2$ but without significance in cell therapy groups [22]. Theoretically, angiogenesis and vascular repair induced by cell therapy should be a relatively longer-term effect compared to direct endovascular revascularization. Because cells need to be mobilized, adhere to target ischemic tissue, then proliferate, and differentiate to form new vessel structure or vessel wall, all improvement due to cell therapy should not be immediate. Meanwhile, for the limited generation of proliferation and survival duration in diabetic tissue environment of autologous cells, the therapeutic effect should be maintained for restricted period.

Immunoregulation of progenitor/stem cells induced by paracrine manner is another often underestimated factor that contributes to the reperfusion of ischemic area in PAD patients. EPCs are reported to secrete series cytokines and chemokines such as VEGF, HGF, MCP-1, IL-1β, IL-6, IL-8, SDF-1, TGF-β, TNF-α, and M-CSF, inducing critical roles of cell proliferation, migration, tubule formation, homing, and matrix degradation [23].

IL-6 mediates activation, growing, and differentiation of T cells. It stimulates the proliferation and fever response and also induces the production of acute phase proteins with IL-1 [24]. IL-6 can be evaluated as a proinflammatory factor and represented inflammatory level. Researchers have proved that IL-6 levels at the diabetic persons are significantly higher than the healthy ones and this altitude may be a risk factor for the complications like atherosclerosis [25, 26]. In our study, we detect a high level of serum IL-6 concentration in diabetic PAD patients, but after cell treatment, IL-6 concentration in $CD133^+$ group remained at lower level than in control group within four weeks (P = 0.026, 0.000, and 0.001 at 1, 2, and 4 weeks), indicating a profounder anti-inflammatory regulation effect with the infusion of $CD133^+$ cells.

VEGF activates o EPCs and results in new blood vessel formation [27]. The potential of VEGF to induce neovascularization is well established both experimentally and clinically [28]. In our research, we also test the serum VEGF level before and after the treatment. The result showed persistent rising of VEGF in $CD133^+$ group more than in control since the first week (79.01 ± 7.75 versus 74.65 ± 6.74 pg/mL, P = 0.034) after cell infusion and lasted for at least 4 weeks (82.45 ± 4.74 versus 74.07 ± 8.00 pg/mL, P = 0.000, at 2 weeks;

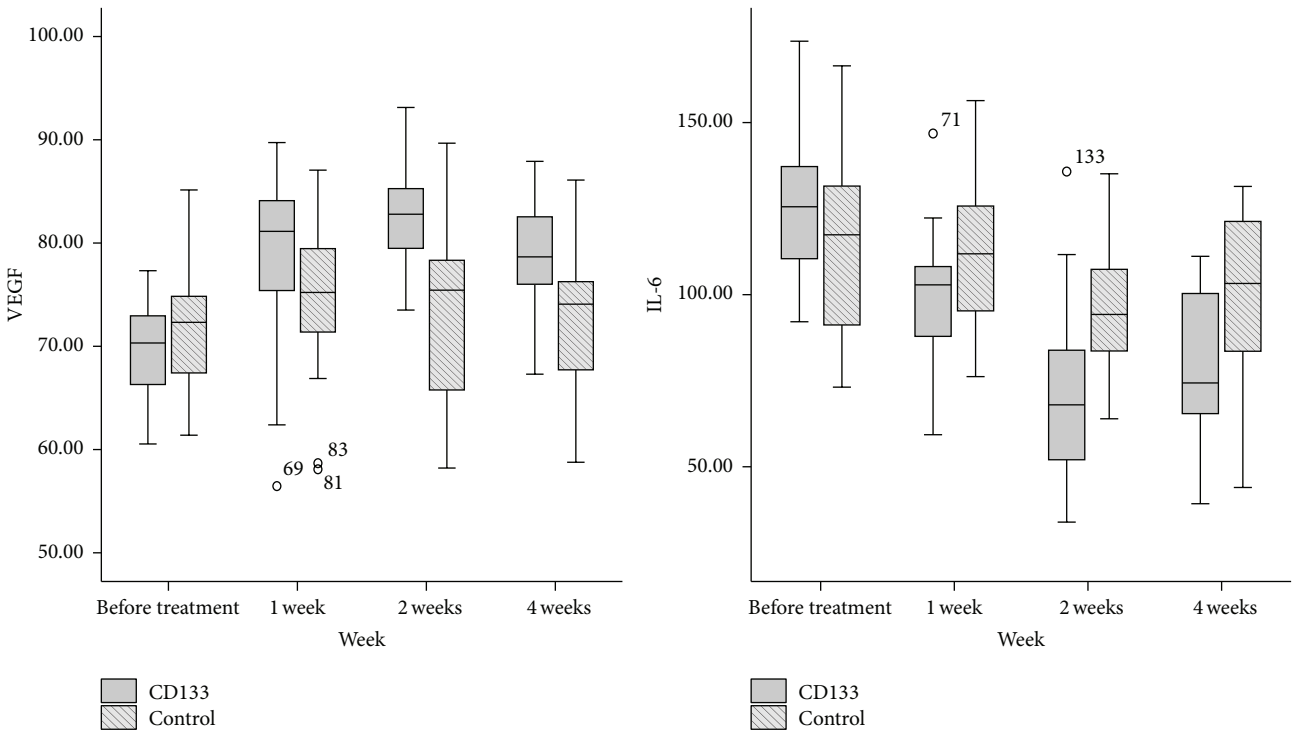

Figure 3: Serum VEGF and IL-6 level change within 4 weeks after treatment. $CD133^+$ group had a significant increasing VEGF and decreasing IL-6 level than the control since the first week after treatment, and this significance seemed to become weaker from the 4th week after treatment. "∘" represents mild outliers in boxplot, and the small numbers beside it represent the case numbers.

78.61 ± 5.04 versus 72.88 ± 6.64 pg/mL, $P = 0.001$, at 4 weeks), indicating a continuous proangiogenesis effect with the infusion of $CD133^+$ cells.

From the change of VEGF and IL-6, we also firstly found a reducing trend of proangiogenesis and anti-inflammatory regulation function at 4 weeks after the cells infusion (Figure 3). This phenomenon may be caused by the life and proliferation of upper threshold of autologous $CD133^+$ cell, which suggest that cell therapy should be repeated at fixed interval according to cell life span.

In conclusion, despite the nonrandomized, no-blinded design and relative small size, results concluded from the study provide initial evidences of the efficacy and safety of autologous $CD133^+$ cells treatment for diabetic PAD, as well as the immunoregulatory impact of VEGF and IL-6 paracrine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiaoping Zhang, Weishuai Lian, and Wensheng Lou contributed equally to this work.

Acknowledgments

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Research Article

Downregulation of the Yes-Associated Protein Is Associated with Extracellular Matrix Disorders in Ascending Aortic Aneurysms

Haiyang Li,[1,2,3,4,5,6] Wenjian Jiang,[1,2,3,4,5,6,7] Weihong Ren,[2,3,6,7] Dong Guo,[1,2,3,4,5,6] Jialong Guo,[1,2,3,4,5,6] Xiaolong Wang,[1,2,3,4,5,6] Yuyong Liu,[1,2,3,4,5,6] Feng Lan,[2,3,6,7] Jie Du,[1,2,3,6,7] and Hongjia Zhang[1,2,3,4,5,6,7]

[1]*Department of Cardiac Surgery, Beijing Anzhen Hospital, Capital Medical University, Beijing 10029, China*
[2]*Beijing Institute of Heart, Lung and Blood Vessel Diseases, Beijing 10029, China*
[3]*Beijing Lab for Cardiovascular Precision Medicine, Beijing 10029, China*
[4]*The Key Laboratory of Remodeling-Related Cardiovascular Disease, Ministry of Education, Beijing 10029, China*
[5]*Beijing Aortic Disease Center, Cardiovascular Surgery Center, Beijing 10029, China*
[6]*Beijing Engineering Research Center for Vascular Prostheses, Beijing 10029, China*
[7]*Beijing Collaborative Innovation Center for Cardiovascular Disorders, Beijing 10029, China*

Correspondence should be addressed to Jie Du; jiedubj@126.com and Hongjia Zhang; zhanghongjia722@hotmail.com

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Previous studies indicate that extracellular matrix (ECM) disorders lead to the apoptosis of Vascular Smooth Muscle Cells (VSMCs), which impairs the aortic wall by reducing the generation of elastic fibers, and ultimately result in ascending aortic aneurysm. The critical role of the Yes-associated protein (YAP) has been elucidated in cardiac/SMC proliferation during cardiovascular development. However, the association of YAP expression and extracellular matrix disorders in ascending aortic aneurysms is not clear. Here, we present for the first time that the downregulation of YAP in VSMCs is associated with ECM disorders of the media in ascending aortic aneurysms. We found that aortic ECM deteriorated with increased apoptotic VSMCs. Moreover, expression of YAP was dramatically reduced in the aortic walls of patients with ascending aortic aneurysms, while the normal aortic samples exhibited abundant YAP in the VSMCs. These results suggest that downregulation of YAP leads to apoptosis of VSMCs, which are essential for the homeostasis of the aortic wall. The resultant ECM disorders affect aortic structure and function and contribute to the development of ascending aortic aneurysms. In summary, through assessment of clinical samples, we revealed the association between downregulation of YAP in VSMCs and the development of ascending aortic aneurysms, providing new insight into the pathogenesis of this disease.

1. Introduction

Ascending aortic aneurysm is a high-risk vascular disorder, which presents as a dilatation or bulging of the ascending aorta that has a variety of causes, such as damage to the aortic wall. As the ascending aortic aneurysm progresses, the weakened aortic wall is unable to withstand the pressure of blood flow and ultimately ruptures [1]. Thus, ascending aortic aneurysms are among the most fatal cardiovascular diseases. At present, both the pathogenesis and pathophysiology of ascending aortic aneurysms are not completely clear, but Vascular Smooth Muscle Cells (VSMCs) are recognized as the most important factor in the development of ascending aortic aneurysms [1, 2]. Existing studies suggest that aortic disorders are likely to occur when the aortic wall cannot handle the stress [1, 3–5]. Mechanical disruption, which is related to disorders of the extracellular matrix (ECM), plays an important role in aortic wall remodeling and induces apoptosis of VSMCs [6, 7].

The Yes-associated protein (YAP), which is involved in the regulation of cell proliferation and apoptosis, is one of the most significant cellular signaling participants *in vivo* [4, 8]. Recent studies have shown that YAP regulates the expression of genes involved in cell proliferation and cell cycle and

apoptosis at the transcriptional level [8], and YAP promotes cell division and maintenance of cell life [9]. The functional role of YAP has also been implicated in cardiac/SMC proliferation during cardiovascular development [10]. According to our previous study, YAP plays a key role in hypertrophic cardiomyopathy [11]. Additionally, abnormal aorta is observed in smooth muscle-specific *YAP*-KO mice [10]. Therefore, we asked whether YAP is also involved in the extracellular matrix disorders in ascending aortic aneurysms through regulation of the functions of VSMCs. Our results showed that YAP was downregulated in human ascending aortic aneurysm samples, and increased apoptosis of VSMCs was observed. We hypothesized that the downregulation of YAP is related to damage to the ECM in ascending aortic aneurysms, which has been implicated in tumorigenesis [12, 13], and led to the increased apoptosis of VSMCs. We also revealed that the occurrence of aortic diseases such as ascending aortic aneurysms is closely related to VSMC apoptosis, which is consistent with previous studies [14–16]. In summary, our findings show that downregulated YAP is associated with ECM disorders and contributes to the development of ascending aortic aneurysms.

2. Materials and Methods

2.1. Clinical Samples. The ethics committee of our institute approved the use of human aortic tissue samples. Normal human aortic samples were obtained from heart transplantation donors. Human ascending aortic aneurysm samples were obtained from patients undergoing surgical replacement of the ascending aorta. Patients with genetic aortic diseases, such as Marfan's, Turner, Loeys-Dietz, or Ehlers-Danlos syndrome, were excluded. All tissue samples were collected immediately after they were resected during the surgery. The clinical information of the patients is shown in Table 1.

2.2. Elastin Staining. For elastin staining of the human aorta, an established method was performed using an elastic fiber staining kit (Maixin Bio) [17]. To remove the paraffin, sections were treated with xylene and rehydrated. Then, the following procedure was carried out: sections were incubated for 5 min in Lugol's iodine solution, washed with PBS, incubated with sodium thiosulfate for 5 min, washed with PBS and 70% ethanol, incubated with aldehyde-fuchsin for 10 min, and then incubated with acid Orange G for seconds.

2.3. Western Blot Analysis. Human aortic samples were harvested and stored at less than −80°C. Protein was extracted using a protein extraction kit containing protease inhibitor and Protein Phosphatase Inhibitor Cocktail. Equal amounts of protein extracts (40 μg/lane) were separated with a 10% SDS-PAGE gel. The expression levels of the GAPDH and YAP proteins were probed by incubation with the primary antibodies anti-GAPDH (1 : 2000 dilution, Sigma-Aldrich) and YAP (1 : 1000 dilution, Cell Signaling), respectively, for over 6 hours at 4°C, followed by incubation with IR dye-conjugated secondary antibodies (1 : 5000, Rockland Immunochemicals, Gilbertsville, PA) for 1 hour. Then, the results were analyzed using an Odyssey infrared imaging system (LI-COR Biosciences Lincoln, NE).

2.4. Histology and Immunohistochemistry. Human aortic samples were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm intervals. Immunohistochemical staining was performed using established methods [17]. To remove the paraffin, sections were treated with xylene and rehydrated. Then, they were incubated with 3% H_2O_2 for 10 min at room temperature and washed 3 times with phosphate-buffered saline (PBS). After blocking with serum for 30 min, the sections were incubated with primary antibodies against YAP (1 : 1000 dilution, Cell Signaling), α-smooth muscle actin (α-SMA, 1 : 500 dilution, Sigma), Bcl-2 (1 : 1000 dilution, Cell Signaling), and cleaved caspase-3 (1 : 300 dilution, Cell Signaling), followed by incubation with the ChemMate EnVision System (Dako). ImageProPlus 3.0 (ECIPSE80i/90i) was used to capture the images and analyze the results. For cryostat sections, human and mouse aortic samples were fixed in 4% paraformaldehyde, embedded in optimum cutting temperature (OCT) compound, frozen in liquid nitrogen, and sectioned at 5 μm intervals. DeadEnd Fluorometric TUNEL (Promega) was used to detect the apoptotic cells. Apoptotic VSMCs were detected by TUNEL and α-SMA double staining before confocal fluorescence microscopy analysis (Leica Microsystems).

2.5. Statistical Analysis. Statistical analysis of the data was performed with a two-tailed Student's t-test. The data are presented as mean ± SEM, with the exception of the luciferase assay data in which mean ± SD is shown. $^{*}p < 0.05$ or $^{**}p < 0.01$ denotes statistical significance.

3. Results

3.1. Aortic Wall Samples of Patients with Ascending Aortic Aneurysms Exhibit Damage to the ECM and Increased Apoptosis of VSMCs. To investigate the pathogenesis of ascending aortic aneurysms, human aortic wall samples from 8 patients undergoing ascending aorta replacement and age-matched heart transplantation donors were collected at our institute. The clinical information of the patients is shown in Table 1. There were 5 males and 3 females with a mean age of 61 ± 8.02 (range: 52 to 72) years. The mean diameter of the ascending aorta from patients with ascending aortic aneurysms calibrated by preoperative echocardiography was 55.75 ± 6.16 mm, and the mean diameter of the aortic sinus was 43.25 ± 7.89 mm. Two patients suffered simultaneously from aortic bicuspid. Various patients underwent different surgeries such as the Bentall procedure and ascending aorta replacement sufficient for the level of aortic involvement, as well as solutions for complications, including coronary heart disease and dilation of the aortic arch and Stanford type B aortic dissection. Computed tomography and intraoperative images showed typical ascending aortic aneurysms (Figure 1(a)). For example, in Figure 1 an obvious ascending aortic aneurysm was shown with a diameter of over 60 mm. Derangement of VSMCs and dissection of elastic lamellae were observed by elastin staining in

Table 1: Ascending aortic aneurysm patients' information related to the aortic tissue used in the study.

Number	Age	Gender	Diameter of ascending aorta (mm)	Diameter of aortic sinus (mm)	Aortic insufficient	Aortic bicuspid	Complications	Surgery
1	65	Male	55	55	Moderate	No	Stanford type B aortic dissection	Ascending aorta replacement + total arch replacement by a tetrafurcate graft and stented elephant trunk implantation
2	70	Male	52	52	Mild	No	Coronary heart disease and dilation of aortic arch	Ascending aorta replacement + partial aortic arch + CABG
3	59	Male	53	50	Severe	No	None	Bentall
4	74	Male	69	41	Mild	No	Coronary heart disease and dilation of aortic arch	Ascending aorta replacement + partial aortic arch + CABG
5	52	Female	59	40	Moderate	No	Dilation of aortic arch	Bentall + partial aortic arch replacement
6	60	Female	51	37	Severe	No	Mitral regurgitation	Bentall + MVR
7	54	Female	57	36	Severe	Yes	None	Bentall
8	54	Male	50	35	Moderate	Yes	Stanford type B aortic dissection	Ascending aorta replacement + total arch replacement by a tetrafurcate graft and stented elephant trunk implantation

CABG: Coronary Artery Bypass Graft; MVR: Mitral Valve Replacement.

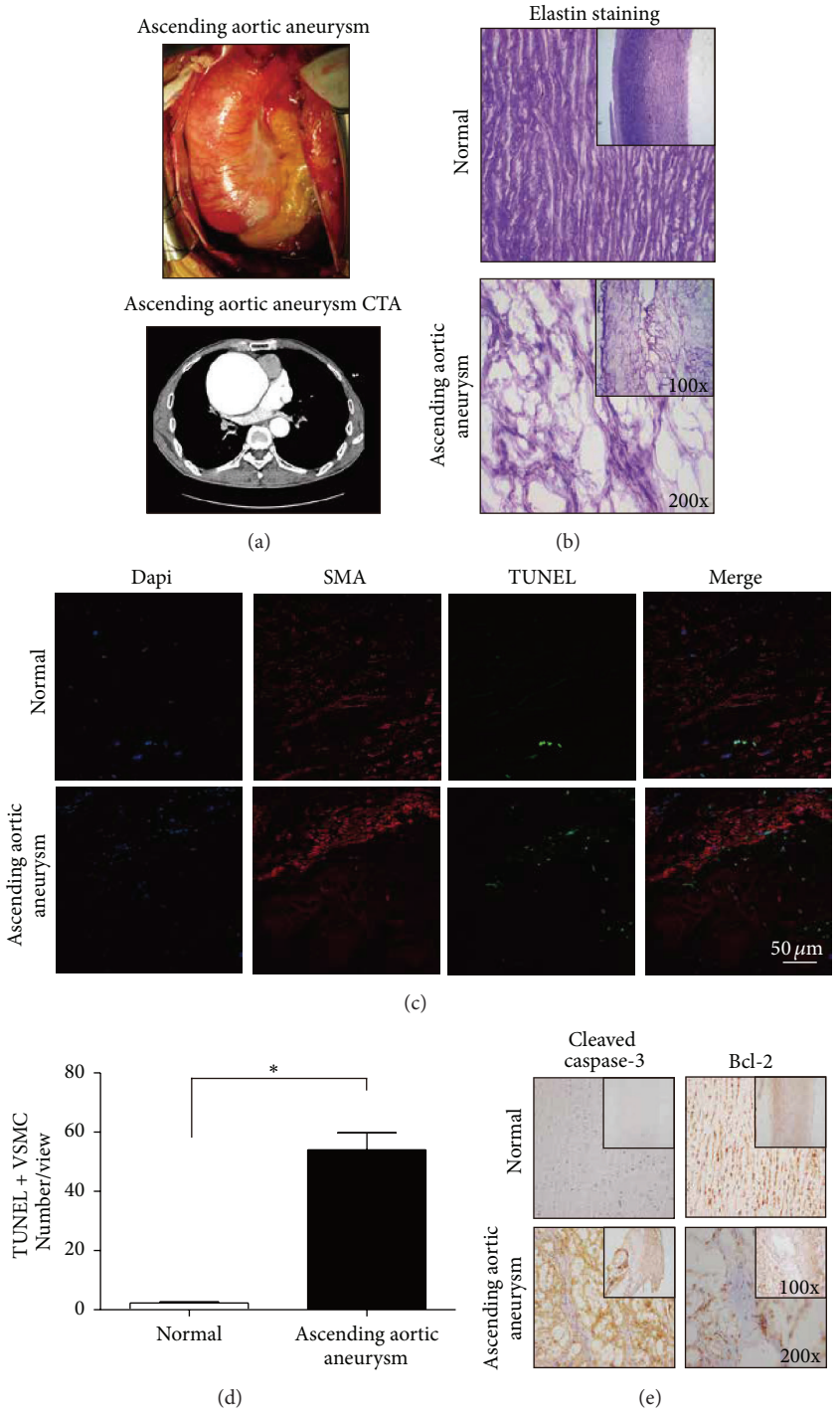

Figure 1: (a) Intraoperative images and CTA showing the enlarged ascending aorta in an ascending aortic aneurysm. (b) Elastin staining showed obvious derangement of tissue structure of the ascending aorta in ascending aortic aneurysm. (c, d) Confocal fluorescence microscopy showed that the number of TUNEL and α-SMA double-staining cells was clearly increased in the ascending aortic walls of ascending aortic aneurysms ($^{**}P = 0.0014$). (e) Immunohistochemistry showed that the cleaved caspase-3 staining was strong and Bcl-2 staining was weak in the ascending aortic walls of ascending aortic aneurysms relative to normal.

patient samples (Figure 1(b)). Through confocal fluorescence microscopy analysis of TUNEL and α-SMA double staining, the increase of VSMC apoptosis in patients with ascending aortic aneurysms was clear compared with the normal control samples (Figure 1(c)). Although there were also some apoptotic cells in the aortic walls of normal human ascending aortas, they were rarely VSMCs (Figure 1(c)). Based on counting the number of apoptotic VSMCs in different views, VSMC apoptosis in the aortic walls of patients with ascending aortic aneurysms was found to be significantly higher than that of

the normal human control samples (Figure 1(d), $p = 0.0014$). We also detected the apoptosis of VSMCs in patients with ascending aortic aneurysms by immunohistochemical staining of cleaved caspase-3 and Bcl-2 (Figure 1(e)). Immunohistochemistry revealed that cleaved caspase-3 staining was strong and Bcl-2 staining was weak in the ascending aortic walls of ascending aortic aneurysms relative to normal, both of which suggested increased apoptosis in the aortic walls of patients with ascending aortic aneurysms.

3.2. YAP Expression Was Reduced in the Aortic Walls of Patients with Ascending Aortic Aneurysms. To further assess the expression of YAP, we performed Western blotting and immunohistochemical staining to evaluate the abundance of the protein. We found a significant reduction of YAP at the protein level in patients with ascending aortic aneurysms. Western blotting revealed that the expression of YAP decreased in the ascending aortic walls of patients with ascending aortic aneurysms compared with that of normal controls (Figure 2(a)), and the results analyzed using the Odyssey infrared imaging system revealed that this difference was significant (Figure 2(b), $p = 0.0047$). Furthermore, immunohistochemistry showed that YAP was weak in the aortic walls of ascending aortic aneurysms relative to normal, which also suggested the downregulation of YAP in the aortic walls of ascending aortic aneurysms (Figure 2(c)). The confocal fluorescence microscopy analysis of YAP and α-SMA double staining showed that the expression of YAP was reduced in VSMCs from the aortic walls of patients with ascending aortic aneurysms (Figure 2(d)), which revealed the downregulation of YAP in VSMCs of ascending aortic aneurysms.

4. Discussion

Our findings regarding the changes of the aortic wall are consistent with those reported by previous studies [18–20] that also suggest the significance of VMSC apoptosis in the pathology of aortic medial degeneration. Given the difficulty of obtaining clinical ascending aortic aneurysm samples, the pathogenesis has not yet been fully elucidated, and most of the current hypotheses come from the research of abdominal aortic aneurysms [21, 22]. However, due to the significant differences between ascending aortic aneurysms and abdominal aortic aneurysms in terms of epidemiology, embryonic origin, genetic development, hemodynamics, and pathology, these hypotheses of etiology speculated from the research results of abdominal aortic aneurysms cannot completely clarify the pathogenesis of ascending aortic aneurysms [23]. In recent years, with the development of various experimental techniques, the study of the etiology of ascending aortic aneurysms has gradually increased. The existing research results show that the occurrence of ascending aortic aneurysms is a result of the interaction of the ECM and VSMCs: Changes in the ECM will induce the apoptosis of VSMCs, influence the function of the aortic wall, reduce the generation of elastic fibers, and then cause the expansion of the ascending aorta [24–26]. The occurrence of many aortic diseases is closely related to the apoptosis of aortic VSMCs [14–16, 27]. In animal aortic injury models, the apoptosis of aortic VSMCs is observed [28, 29]. However, there is no known mechanism of VSMC apoptosis in the aortic walls of patients with ascending aortic aneurysms.

The wall of the aorta contains three layers. The media is made up of collagen, VSMCs, and roughly 50 elastic laminas, which can be highly stretched [30]. The etiology of ascending aortic aneurysms is mainly histopathology and genetic factors, which suggest that preexisting weakness of the aortic media is the basis of the occurrence of ascending aortic aneurysms [1, 31]. Media degeneration refers to cystic degeneration or necrosis of elastic fibers, including rupture of the elastic lamina and apoptosis of VSMCs [32, 33]. Media degeneration also generates ECM mechanical stress. Moreover, apoptosis of VSMCs could be induced by cyclic stretch, which is thought to be a simulation of mechanical stress [17]. Media degeneration, including rupture of the elastic lamina and apoptosis of VSMCs, has been reported in human ascending aortic aneurysms [31, 32]. Consistent with this, aortic wall samples of human ascending aortic aneurysms displayed disrupted elastic lamellae, which indicates medial degeneration in our study. Upon costaining of TUNEL with α-SMA, the increase of VSMC apoptosis in the aortic walls of patients with ascending aortic aneurysms is clear compared with the normal control samples. We also detected apoptosis of VSMCs by performing staining of cleaved caspase-3, and we found results similar to those obtained by the costaining of TUNEL with α-SMA.

Some members of the Hippo-YAP pathway have been reported to have the ability to induce apoptosis in rat aortic VSMCs [34]. In this study, we observed a similar phenomenon that downregulated YAP was accompanied by VSMC apoptosis and was associated with ECM disorders in ascending aortic aneurysms. The above result resembles those reported by previous studies: Because mechanical stress caused by differences in the ECM is crucial to regulating the expression of YAP [13], YAP will be inhibited when cells are cultured on soft matrix [35]. Clearly, disrupted elastic lamellae with variable width in STAAD soften the ECM, which might induce the downregulation of YAP.

There are several limitations of the present study. Most obviously, the results are only the description of phenomenon in the ascending aortic aneurysm. However, given the precious value of the aortic wall samples of human ascending aortic aneurysms, this is the most realistic representation of ascending aortic aneurysms. Further studies to elucidate the mechanisms of ascending aortic aneurysms *in vivo* and *in vitro* will be continued in the future; however, the results of this paper provide the basis for such mechanistic studies. Furthermore, due to the low numbers of samples included in the present study, we may have overestimated the effect of the downregulation of YAP in VSMCs. Most notably, the role of YAP in the apoptosis of VSMCs of the media in ascending aortic aneurysms as observed in this study needs to be examined further.

5. Conclusions

Our study is the first to show that the downregulation of YAP in VSMCs is closely associated with ECM disorders of

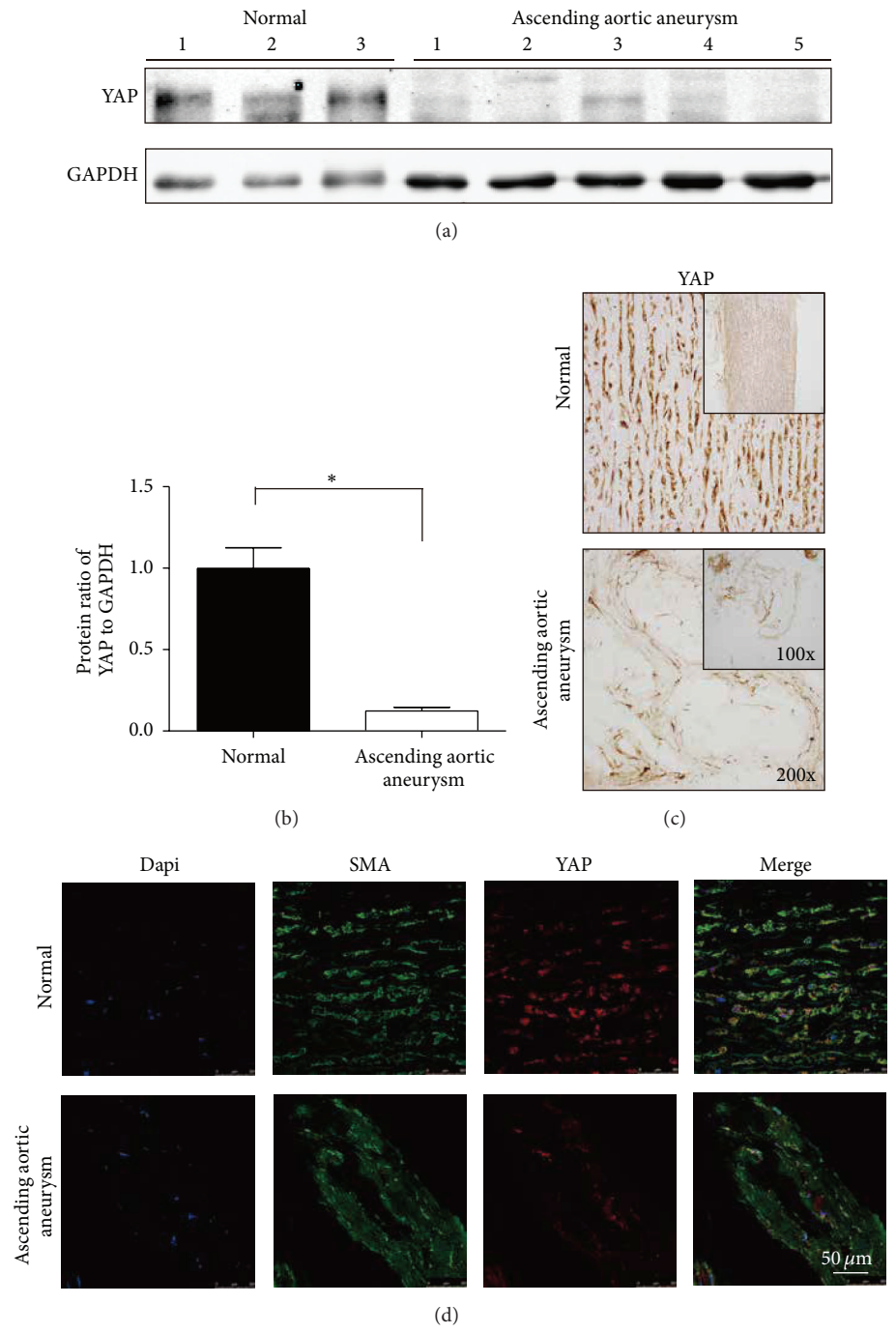

FIGURE 2: (a, b) Western blotting showed that expression of YAP decreased significantly in the ascending aortic walls of patients with ascending aortic aneurysms compared to that of normal controls ($^{**}p = 0.0047$). (c) Immunohistochemistry showed that YAP was weak in the ascending aortic walls of ascending aortic aneurysms relative to normal. (d) Confocal fluorescence microscopy showed that YAP and α-SMA double-staining cells were reduced in ascending aortic aneurysms.

the media in ascending aortic aneurysms. The downregulated YAP accompanied by the alteration in mechanical stress caused by ECM disorders in the aortic wall is essential for aortic structure and function, and apoptosis of VSMCs following the downregulation of YAP may contribute to the pathogenesis of ascending aortic aneurysms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Haiyang Li and Wenjian Jiang contributed equally to this study.

Acknowledgment

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Research Article

Residual Dyslipidemia Leads to Unfavorable Outcomes in Patients with Acute Coronary Syndrome after Percutaneous Coronary Intervention

Bin Que, Chunmei Wang, Hui Ai, Xinyong Zhang, Mei Wang, and Shaoping Nie

Emergency and Critical Care Center, Beijing Anzhen Hospital, Capital Medical University, Beijing Institute of Heart Lung and Blood Vessel Disease, Beijing 100029, China

Correspondence should be addressed to Shaoping Nie; spnie@126.com

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Background. The present study aimed to evaluate the prevalence and prognosis of residual lipid abnormalities in statin-treated acute coronary syndrome (ACS) patients after percutaneous coronary intervention (PCI). *Subjects and Methods*. A total of 3,047 ACS patients who underwent PCI and received statin therapy were included. Plasma concentrations of LDL-C, HDL-C, and TG were measured. For the follow-up study, major adverse cardiovascular cerebrovascular events (MACCE; including total death, cardiovascular death, myocardial infarction, and revascularization) were documented. *Results*. A total of 93.14% of all individuals were followed up for 18.1 months (range, 0–29.3 months). Of all 3,047 patients, those with a suboptimal goal were 67.75%, 85.85%, and 33.64% for LDL-C, HDL-C, and TG levels, respectively. Multiple Cox regression analysis revealed there were significant increases in cumulative MACCE of 41% (HR = 1.41, 95% CI [1.09–1.82], $p = 0.008$), and revascularization of 48% (HR = 1.48, 95% CI [1.10–1.99], $p = 0.01$) in low HDL-C patients with ACS after PCI, but not the high TG group at the end of study. *Conclusions*. Our results showed there is high rate of dyslipidemia in Chinese ACS patients after PCI. Importantly, low HDL-C but not high TG levels are associated with higher MACCE and revascularization rates in ACS patients after PCI.

1. Introduction

According to a WHO survey, approximately 4 million people will die of cardiovascular diseases (CVD) in China in 2020. As one of the main risk factors of CVD, dyslipidemia has been widely treated to lower CVD morbidity and mortality. However, accumulating evidence has shown that lipid-lowering treatment with high-intensity statins decreases LDL-C by 20%–30% but only results in 24%–42% reduction of main coronary adverse events [1–3]. In patients treated with statins, residual dyslipidemia occurs when low high-density lipoprotein cholesterol (HDL-C) levels and/or high triglyceride (TG) levels remain. It has been noted that the prevalence of residual dyslipidemia after statin treatment, manifesting as high low-density lipoprotein cholesterol (LDL-C), high TG, or low HDL-C, is high [4–6]. Sirimarco et al. reported that the presence of atherogenic dyslipidemia in subjects with stroke receiving statin therapy was associated with higher residual cardiovascular risk [7]. A cross-sectional trial in China that included 25,697 patients treated with lipid-lowering agents showed that up to 38.5% of patients did not achieve their therapeutic goal. Moreover, 10.4% of very high-risk patients and 11.1% of high-risk patients who attained the LDL-C goal failed to attain non-HDL-C goals [4].

This prospective study was performed to examine the prevalence of residual dyslipidemia in acute coronary syndrome (ACS) patients who underwent percutaneous coronary intervention (PCI) after statin therapy and to evaluate the effect of residual dyslipidemia on major cardiovascular events after 1 year of follow-up.

2. Patients and Methods

2.1. Patients. Patients who had symptoms of ACS and underwent PCI in Beijing Anzhen hospital (from January 1, 2010,

to January 1, 2013) were eligible for this study. Coronary angiography was performed and analyzed to include patients who had either single-vessel disease or multivessel disease. Multivessel disease was defined as ≥50% angiographic diameter stenosis of ≥2 epicardial coronary arteries. Patients with severe congestive heart failure on admission (New York Heart Association III or IV), advanced tumors, or immunologic diseases were excluded. The protocol and consent form were approved by the institutional review board of Beijing Anzhen Hospital. All subjects signed the consent form. Characteristics of all subjects were documented, including age, sex, body weight, height, blood pressure, smoking, and diabetes. All patients enrolled were given optimal medical therapy according to the American Heart Association/American College of Cardiology Foundation "Secondary Prevention and Risk Reduction Therapy for Patients with Coronary and Other Atherosclerotic Vascular Disease" unless contraindicated, including aspirin, anticoagulation if indicated, angiotensin-converting-enzyme inhibitor/angiotensin II receptor blockers, beta-receptor blockers, and statins.

2.2. Follow-Up Study. After 3 months of statin treatment, ACS patients were required to attend at outpatient visit to measure their lipid levels. A consecutive series of 3,047 ACS patients treated with statins for at least 3 months were enrolled. Lipid parameters, including total cholesterol (TC), LDL-C, HDL-C, TG, uric acid, creatinine, and high-sensitivity C-reactive protein levels, were measured and collected. The follow-up study was executed by trained personnel using a standardized questionnaire at 6 months and 1 year of follow-up. Major adverse cardiovascular cerebrovascular events (MACCE) were defined as cardiovascular death, reinfarction, revascularization, and stroke. A total of 2,838 (93.14%) patients were successfully followed. A total of 2,639 (99.6%) patients were followed up by phone, 166 patients were followed up in the clinic, and 33 patients were followed up in the hospital.

2.3. Statistical Methods. All analyses were performed with Stata, version 11.0 software. Patients who had no lipid parameters were not included in the lipid analyses. Continuous variables were reported using descriptive statistics (mean ± standard deviation [SD] or median with Q1–Q3 interquartile range). For categorical variables, mean ± SD was reported and comparisons were made using the chi-square or Fisher's exact test. Kaplan-Meier analysis was performed to evaluate survival. Prognosis of patients after PCI was analyzed using a Cox proportional hazard model. Results were considered significant or not significant if $p < 0.05$ or ≥0.05, respectively.

3. Results

3.1. Overall Subject Characteristics. A total of 3,047 patients were enrolled in the present study, and 2,838 (93.14%) had complete follow-up information. The median follow-up time was 543 days; the mean follow-up period was 537 days. The mean age of all 3,047 ACS patients was 59.6 ± 10.6 years, of whom 23.0% were female, 34.1% were smokers, 61.8% had hypertension, and 26.6% had diabetes mellitus.

3.2. Residual Dyslipidemia in Patients after PCI

3.2.1. Low HDL-C. The goal of HDL-C level was >40 mg/dL in men and >50 mg/dL in women. Of the 3,047 statin-treated patients, 67.65% had an HDL-C level lower than the goal; among these, 73.1% were male. In the LDL-C goal group, 76.33% of subjects sustained a lower HDL-C than the normal group. The percentage of women in the normal HDL-C level group was lower than that of the normal group (16.5% versus 26.9%, $p < 0.01$).

The percentage of women (16.5% versus 26.9%), age (60.6 ± 10.4 versus 59.1 ± 10.4 years), TC level (189.6 ± 45.5 versus 167.4 ± 40.6 mg/dL), LDL-C level (115.7 ± 37.7 versus 100.8 ± 32.5 mg/dL), TG level (123 [90–171] versus 145 [107–201] mg/dL), glucose level (107.3 ± 35.1 versus 111.4 ± 41.1 mg/dL), percentage of patients with hypertension (58.6% versus 63.8%), and proportion of patients with diabetes (22.5% versus 28.9%) were higher in low HDL-C group than in the normal HDL-C group ($p < 0.01$) (Table 1).

3.2.2. Elevated TG. High TG levels were defined as those >200 mg/dL. Of the statin-treated patients, 42.83% had higher TG levels than the goal, and 76.4% were male. The percentage of women in the normal TG group was similar to the higher TG group (22.7% versus 23.5%, $p > 0.05$). The age (60.8 ± 10.5 versus 58.1 ± 10.6 years), BMI (189.6 ± 45.5 versus 167.4 ± 40.6 mg/dL), TC level (166.3 ± 39.3 versus 187.9 ± 46.4 mg/dL), HDL-C level (0.6 ± 9.1 versus 38.3 ± 9.1 mg/dL), LDL-C level (101.6 ± 33.8 versus 108.7 ± 35.8 mg/dL), glucose level (106.1 ± 36.7 versus 115.2 ± 41.5 mg/dL), estimated glomerular filtration rate (eGFR; 86.6 ± 26.1 versus 84.4 ± 5.2 mL/min), and proportion of patients with diabetes (22.7% versus 31.7%) were significantly different in the higher TG group than in the normal HDL-C group ($p < 0.01$) (Table 1).

3.2.3. LDL-C Goal Attainment. Patients with ACS after PCI belong to the highest cardiovascular disease risk category and have a target LDL-C of <70 mg/dL. Of the 3,047 statin-treated patients, 14.15% had levels lower than the goal; 82.6% of whom were male. LDL-C levels remained higher than goal for 85.85% of subjects, 76% of whom were male.

The two groups were similar regarding age (59.8 ± 10.5 versus 59.6 ± 10.7 years), glucose level (109.7 ± 42.2 versus 109.9 ± 38.5 mg/dL), percentage with hypertension (64.5% versus 61.4%), and proportion with diabetes (30.2% versus 26%). The percentage of women (24% versus 17.4%, $p < 0.05$), eGFR (88.4 ± 31.1 versus 85.4 ± 24.7 mL/min, $p < 0.05$), and proportion of smokers (29.5% versus 34.9%, $p < 0.05$) were higher in the target LDL-C group than in group not meeting the target LDL-C level (Table 1).

3.3. The Relationship between Lower HDL-C and Adverse Events. Multiple Cox regression analysis revealed that there were significant 41% increase in cumulative MACCE (hazard ratio [HR] = 1.41, 95% CI [1.09–1.82], $p = 0.008$) and 48%

Table 1: Patient characteristics, risk categories, and lipid parameters.

	All patients	LDL-C at goal	LDL-C not at goal	Normal HDL-C	Low HDL-C	Normal TG	High TG
N	3047	431	2616	1127	1920	1742	1305
Age (years)	59.6 ± 10.6	59.8 ± 10.5	59.6 ± 10.7	60.6 ± 10.4	59.1 ± 10.7$^{\#\#}$	60.8 ± 10.5	58.1 ± 10.6††
Female (%)	23.0	17.4	24.0*	16.5	26.9$^{\#\#}$	22.7	23.5
BMI (kg/m^2)	25.8 ± 3.1	25.5 ± 3.1	25.9 ± 3.1*	25.3 ± 3.0	26.1 ± 3.1	25.4 ± 3.1	26.3 ± 3.0††
SBP (mmHg)	128.7 ± 19.3	128.5 ± 19.6	128.7 ± 19.2	129.7 ± 19.1	128.1 ± 19.3$^{\#}$	128.8 ± 19.9	128.6 ± 18.3
DBP (mmHg)	78.3 ± 10.9	78.1 ± 10.6	78.4 ± 11.0	78.6 ± 11.0	78.2 ± 10.9	77.9 ± 10.9	79.0 ± 11.0††
TC (mg/dL)	175.4 ± 43.8	128.7 ± 22.7	183.2 ± 41.5**	189.6 ± 45.5	167.4 ± 40.6$^{\#\#}$	166.3 ± 39.3	187.9 ± 46.4††
HDL-C (mg/dL)	39.6 ± 9.2	36.2 ± 8.6	40.2 ± 9.2**	48.2 ± 8.3	34.9 ± 5.5$^{\#\#}$	40.6 ± 9.1	38.3 ± 9.1††
LDL-C (mg/dL)	104.6 ± 34.8	60.5 ± 8.4	112.1 ± 32.0**	111.5 ± 37.7	100.8 ± 32.5$^{\#\#}$	101.6 ± 33.8	108.7 ± 35.8††
TG (mg/dL)	137 (100–191)	121 (85–176)	140 (103–193)**	123 (90–171)	145 (107–201)$^{\#\#}$	105 (84–126)	204 (172–259)††
FBG (mg/dL)	109.9 ± 39.1	109.7 ± 42.2	109.9 ± 38.5	107.3 ± 35.1	111.4 ± 41.1$^{\#\#}$	106.1 ± 36.7	115.2 ± 41.5††
eGFR (mL/min)	85.7 ± 25.6	88.4 ± 31.1	85.2 ± 24.7*	85.6 ± 25.6	85.7 ± 25.8	86.6 ± 26.1	84.4 ± 25.2†
LVEF (%)	58.3 ± 10.9	58.1 ± 10.6	58.4 ± 11.0	58.6 ± 11.0	58.2 ± 10.9	57.9 ± 10.9	59.0 ± 11.0
Current smoker (%)	34.1	29.5	34.9*	34.1	34.1	33.5	35.0
Hypertension (%)	61.8	64.5	61.4	58.6	63.8$^{\#\#}$	60.4	63.8
Diabetes mellitus (%)	26.6	30.2	26.0	22.5	28.9$^{\#\#}$	22.7	31.7††
Multivessel disease (%)	46.7	46.2	46.7	43.7	44.1	46.9	47.0

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; FBG: fasting blood glucose; eGFR: estimated glomerular filtration rate; LVEF: left ventricular ejection fraction. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LDL-C at goal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with normal HDL-C group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared with normal TG group.

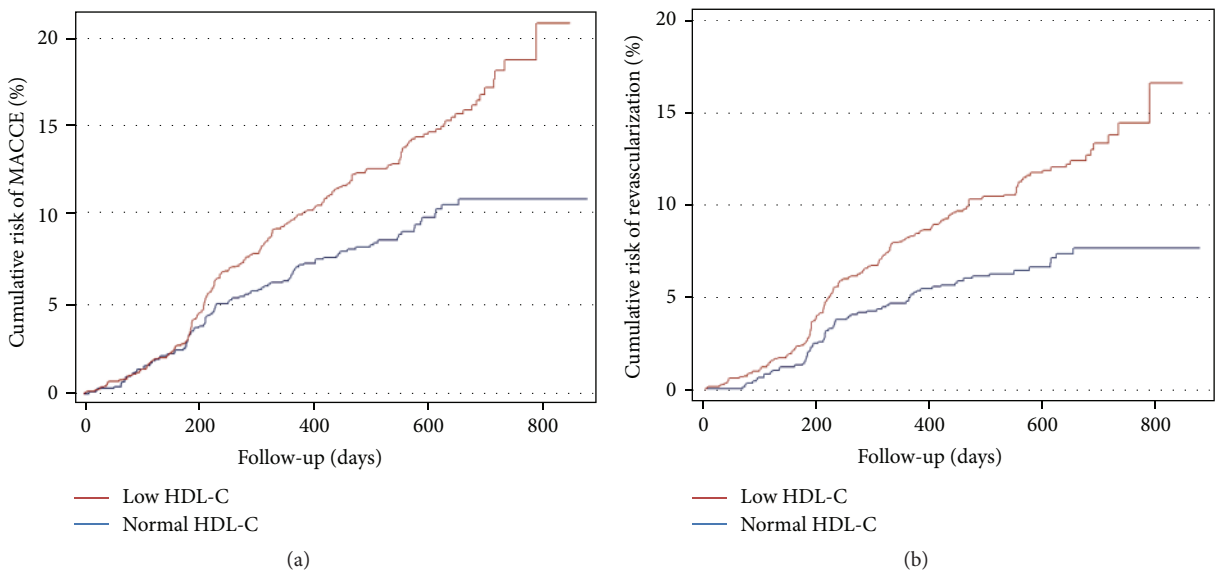

Figure 1: Kaplan-Meier curve of cumulative risk of (a) MACCE (major adverse cardiovascular cerebrovascular events) and (b) revascularization when subjects were grouped according to the HDL-C level.

increase in revascularization (HR = 1.48, 95% CI [1.10–1.99], $p = 0.01$) in lower HDL-C patients with ACS after PCI at the end of follow-up (Figure 1). However, lower HDL-C was not associated with any of the following outcomes: cardiovascular death, total death, myocardial infarction, and stroke (Table 2).

3.4. The Relationship between Higher TG and Adverse Events. Between the higher TG group and lower TG group, respectively, the rate of MACCE was 11.9% versus 10.7%, the death rate was 1.8% versus 1.5%, the cardiovascular death rate was 1.5% versus 1.5%, the revascularization rate was 9.1% versus

Table 2: The comparison of MACCE between normal HDL-C group and low HDL-C group.

	Normal HDL-C (N = 1127)		Low HDL-C (N = 1920)		Risk ratio (HR)	p value
	n	(%)	n	(%)		
MACCE	97	8.6	251	13.1	1.41 (1.09–1.82)	0.008*
All cause death	19	1.7	32	1.7	1.25 (0.64–2.41)	0.50
Cardiovascular death	19	1.7	27	1.4	1.50 (0.62–3.64)	0.372
Revascularization	68	6.0	201	10.5	1.48 (1.10–1.99)	0.01*
Myocardial infarction	5	0.4	11	0.6	0.87 (0.28–2.70)	0.80
Stroke	11	1.0	21	1.1	1.44 (0.65–3.18)	0.37

MACCE: major adverse cardiovascular cerebrovascular events. * $p < 0.05$ compared with Normal HDL-C group.

Table 3: The comparison of cardiovascular events between normal TG group and elevated TG group.

	Normal TG (N = 1742)		Higher TG (N = 1305)		Risk ratio (HR)	p value
	n	(%)	n	(%)		
MACCE	208	11.9	140	10.7	0.88 (0.70–1.12)	0.35
All cause death	32	1.8	19	1.5	0.80 (0.42–1.54)	0.51
Cardiovascular death	27	1.5	19	1.5	1.12 (0.52–2.45)	0.76
Revascularization	158	9.1	111	8.5	1.05 (0.81–1.36)	0.69
Myocardial infarction	11	0.6	5	0.4	0.83 (0.27–2.51)	0.74
Stroke	22	1.3	10	0.8	0.63 (0.29–1.40)	0.26

MACCE: major adverse cardiovascular cerebrovascular events.

8.5%, the rate of MI was 0.6% versus 0.4%, and the stroke rate was 1.3% versus 0.8%. Multiple Cox regression analysis revealed that the increase in TG had no relation with any of the following outcomes: MACCE, total death, cardiovascular death, revascularization, MI, or stroke (Table 3).

4. Discussion

Reports from the Dyslipidemia International Study have shown that there is a considerable prevalence of residual dyslipidemia after statin therapy worldwide as well as in China [4, 8, 9]. The previous national cross-sectional investigation in China showed that 29.1% of 25,697 patients with statin therapy had no lipid abnormalities, of which 51.2% did not have a TC at goal and 38.5% did not have LDL-C at goal according to 2007 Chinese guidelines [10].

However, our present data showed that the prevalence of residual dyslipidemia is even higher in ACS patients after PCI. There were 63.1% and 85.85% ACS patients after PCI not achieving goal HDL-C and LDL-C levels, respectively, after statin treatment for 3 months (Table 1). Up to 76.33% patients who attained the LDL-C goal failed to achieve the HDL-C goal. Even with a goal LDL-C of <100 mg/dL, only 48.7% patients achieved it, which is much lower than patients in Western countries. In a large cohort of patients hospitalized with CAD, about half have admission LDL levels < 100 mg/dL, while more than half the patients have admission HDL levels < 40 mg/dL and <10% have HDL ≥ 60 mg/dL [11]. A multinational survey that evaluated the proportion of patients achieving LDL-C goals according to relevant national guidelines ranging from 47% to 84% across countries. The overall success rate for LDL-C goal achievement was 73%, but only 67% in high-risk patients. However, only 30% of CAD patients with no fewer than 2 risk factors attained the optional LDL-C goal of <70 mg/dL [9]. It was also reported that 39.6% of the 4,335 statin-treated patients had lipid values within desirable levels in France. LDL-C was not at goal more often (51.8%) in higher-risk patients than in all patients overall (37.2%). Also, high-risk patients with LDL-C not at goal had additional lipid abnormalities (low HDL and/or high TG) more frequently (25.6%) than all patients overall (18.4%) [10].

It has been considered as a risk factor of CAD of low HDL-C level according to a 21-year follow-up study [12]. Moreover, a study enrolling 30,0000 subjects had shown that about 10% of patients with either stroke or transient ischemic attack presenting with residual dyslipidemia (low HDL-C and high TG) had increased cardiovascular risk [7]. Similar with this finding, our present data showed significant increases in cumulative MACCE by 41% and revascularization by 48% in lower HDL-C patients (Table 2 and Figure 1), but not high TG patients (Table 3), with ACS after PCI at the end of follow-up. However, a small-sized case-control study (170 cases and 175 controls) that evaluated the contributions of TG and HDL-C levels in coronary heart disease patients found that high TG and low HDL-C levels contribute strongly and synergistically to CAD after the reduction of LDL-C to the guideline-recommended level [13].

However, our study has limitation that the long-time follow-up studies are still needed to determine if there is truly an association between the low HDL-C level and the worse clinical outcome in ACS patients after PCI. In conclusion, our present study showed a considerably high prevalence of residual dyslipidemia in Chinese ACS patients after PCI. In addition, low HDL-C levels after statin treatment were closely associated with clinical outcomes. Moreover, the results from our present study suggest that more effort need to be made to improve the dyslipidemia situation, not only for LDL-C level but also for HDL-C levels, to get better clinical outcomes in ACS patients after PCI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Endovascular Management of Aorta-Iliac Stenosis and Occlusive Disease by Kissing-Stent Technique

Meng Liu[1,2] and Fuxian Zhang[1]

[1]*Department of Vascular Surgery, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China*
[2]*Department of Vascular Surgery, Tianjin Hospital, Tianjin 300211, China*

Correspondence should be addressed to Fuxian Zhang; fuxianvascular@163.com

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Kissing-stenting treatment has been used to treat patients with peripheral artery disease (PAD). However, the long term efficacy of the stenting therapy is not well defined in Chinese PAD patients. To investigate the question, sixty-three PAD patients (37 males and 26 females), aged 66 ± 7.3 years, were analysed in the study. They were featured as claudication (n = 45, 71.4%), rest pain (n = 18, 28.6%), or gangrene (n = 8, 12.7%). In total, 161 stents were applied in aorta-iliac lesions with 2.6 stents for each patient, including 55 self-expanding stents, 98 balloon expandable stents, and 8 covered stents. The success rate of implanting Kissing-stents was 100%. Catheter-directed thrombolysis (CDT) with urokinase was performed in 8 cases (12.7%). The severity of peripheral ischemia was significantly improved, as evidenced by 3.3-fold increase of ankle-brachial pressure index (ABI) after the surgery (P = 0.008). One, three, five, and seven years after surgery, the primary patency rate was 87.3%, 77.4%, 71.1%, and 65.0%, whereas the secondary patency rate was 95.2%, 92.5%, 89.5%, and 85.0%, respectively. No in-hospital mortality was recorded. In conclusion, Kissing-stenting technique for aorta-iliac lesions is safe and effective with lower complications. It is beneficial for aorta-iliac occlusions that are longer than 60 mm.

1. Introduction

Aorta-iliac stenosis and occlusion contribute substantially to limb ischemia in patients with peripheral artery disease (PAD). Conventional treatments include bypass surgery such as aorta-iliac artery bypass, axillofemoral artery bypass, and bilateral femoral artery bypass [1–3]. It has been reported that the percentage of mortality and complications following bypass surgery was about 3–5% and 8–13%, respectively [4]. In recent years, endovascular technics have been developed to be the alternative therapy to PAD. Accumulated data have documented that both mortality and complications of endovascular intervention with Kissing-Technique were significantly reduced compared to traditional surgery in western countries [5, 6]. Nevertheless, the long term efficacy of Kissing-stents in the treatment of Chinese PAD patients remains inconclusive. In addition, whether endovascular intervention has overcome the difficulty of managing bifurcations is not well defined.

To investigate these questions, 63 PAD patients with clear diagnosis of aorta-iliac bifurcation occlusion were analysed in the study. In total, 161 stents were applied in the aorta-iliac lesions in the PAD patients. The success rate of implantation of Kissing-stenting achieved 100%. After stenting, the patients were followed up to 7 years to evaluate the short term and long term patency rates using Kissing-stent technique.

2. Materials and Methods

2.1. The Studying Subjects. PAD patients (n = 63) with a mean age of 66 years who were hospitalized between April 2007 and April 2014 were included in the study. Arteriosclerosis was diagnosed in all patients. Among them, there were 39 smokers (61.9%), 51 cases of hypertension (81.0%), 38 cases of diabetes (60.3%), 30 cases of hyperlipidemia (47.6%), 34 cases of coronary artery disease (54.0%), and 18 cases of cerebrovascular disease (28.6%). All 63 patients were evaluated by duplex ultrasonography, magnetic resonance (MR)

angiography, computerized tomography, and ankle-brachial pressure index (ABI) prior to operation. The study received ethical approval from the competent Institutional Review Boards of Capital Medical University and was performed in accordance with relevant guidelines and regulations in Capital Medical University. All participants provided written informed consent.

All the patients met the criteria of using Kissing-stenting treatment as the following: (1) vascular color Doppler ultrasound showed hypoechoic or admixture-echoic signs at the occlusion sites in common iliac artery occlusions despite absence of severe calcification; (2) no anchoring (landing zone) occurred at aorta-iliac junction or proximal position in common iliac artery for PTA and stent placement; and (3) catheter or the guide wire may pass the segments of aorta-iliac occlusive disease. In parallel, the severity of occlusion was further confirmed by digital subtraction angiography (DSA), indicating 42 patients with no proximal iliac stump and 21 patients with short proximal iliac stump (≤1 cm).

2.2. Catheter-Directed Thrombosis prior to Stenting. In order to achieve higher endovascular angioplasty success rate and favorable patency with low complication rate, preoperative catheter-directed thrombolysis (CDT) with urokinase was administered through a Unifuse catheter (Uni*Fuse Infusion Catheter; AngioDynamics, Queensbury, NY) in patients with long-segment aorta-iliac occlusion (≥60 mm). A total of 750,000 U of urokinase diluted in a 50 mL saline solution was administered to the patients via left brachial artery once per day for 2 days. Heparin (400–600 units/hour) was continuously infused into the patients through the sheath. Plasma level of fibrinogen and activated partial thromboplastin time were examined.

In the study, the covered stent was purchased from Gore VIABAHN (Newark, Delaware, USA) or Fluency Plus (Bard Inc., Germany); the self-expanding stents were from Medtronic (Minnesota, USA) or Boston Scientific (Boston, USA); and the balloon expandable stents were from INVATEC (INVATEC Inc., Minnesota, USA).

2.3. Endovascular Angioplasty by Kissing-Stenting Technique. All Kissing-stents were deployed from the proximal end in the aorta, that is, 1–4 cm above the native aortic bifurcation. All self-expanding stents were to be dilated to reach the diameter of the normal vessel, according to angiogram prior to the treatment. In 19 patients without palpable pulses in CFA, the Kissing-stents were entered through the left brachial artery. After placing the stents, the artery was further reinforced by a balloon with the diameter of 4 mm to enhance blood perfusion. For the rest of the patients, guide wire was applied percutaneously following bilateral CFA punctures. The size of stent and balloon was determined according to the reference vessel diameter in the vicinity of the lesion and the contralateral iliac artery.

After stenting, to prevent thrombosis, the patients were prescribed oral intake of 100 mg aspirin and 75 mg clopidogrel daily for 8 weeks and then lifelong monotherapy with aspirin.

Table 1: General characterization of the studying subjects.

Parameters	PAD patients
N	63
Males (%)	37 (63%)
Age (year)	66 ± 7.3
Smoker (0, 1)	39 (61.9%)
Disease (%)	
Hypertension (0, 1)	51 (81.0%)
Diabetes (0, 1)	38 (60.3%)
Hyperlipidemia (0, 1)	30 (47.6%)
Coronary heart disease (0, 1)	34 (54.0%)
Cerebrovascular disease (0, 1)	18 (28.6%)
Medications	
Aspirin (0, 1)	63 (100.0%)
Clopidogrel (0, 1)	51 (81.0%)
Statins (0, 1)	37 (58.7%)
Features of PAD	
Walking distance (m)	88 ± 2 7
Rest pain (0, 1)	18 (28.6%)
Claudication	45 (71.4%)
Mild	27 (42.9%)
Severe	18 (28.6%)

2.4. Evaluations of Kissing-Stenting Surgery. The patients were followed up to 7 years (interquartile range, 3–84 months). The primary and secondary patency rates were recorded. Technical success was defined as residual stenosis less than 30%, a pressure gradient less than 5 mm Hg, and the increase of ABI greater than 0.1.

Loss of primary patency was diagnosed when ABI index is above 20% or restenosis above 50%. In this situation, a secondary intervention was given to maintain arterial patency.

2.5. Statistical Analysis. Statistical analysis was performed by SPSS software (Version 13.0, United Kingdom). Continuous variables are presented as means and standard deviation and proportional data are presented as number and percentage. Unpaired t-test was used to compare ABI index before and after the stenting treatment. The Kaplan-Meier method was used to estimate primary and secondary patency rates. Statistical significance was defined as P value less than 0.05.

3. Results

3.1. General Characterization of PAD Patients. All 63 patients had the clinical symptoms of ischemic limbs, among which 25 patients had critically ischemic limbs. Claudication occurred in 45 (71.4%) patients. Mild claudication is defined as walking distance less than 200 meters whereas severe claudication is defined as walking distance less than 30 meters. Among 45 patients with claudication, there were 27 patients (42.9%) with mild claudication and 18 patients (28.6%) with severe claudication and the average walking distance was 88 ± 2 meters. Moreover, all the patients with severe claudication were also featured as rest pain and 8 of them had suffered from toe gangrene. The lesion length of occlusion in aorta-iliac artery or iliac artery was 6.0 ± 4.9 cm. The averaged ABI index was 0.28. The general characteristic of the patients is shown in Table 1. Eight patients had occlusions

Table 2: TASC II classification of aorta-iliac lesions.

Type	Criteria
A	(i) Unilateral or bilateral stenosis of CIA (ii) Unilateral or bilateral single short (≤3 cm) stenosis of EIA
B	(i) Short-segment (≤3 cm) stenosis of infrarenal aorta (ii) Unilateral CIA occlusion (iii) Single or multiple stenosis (3–10 cm) involving the EIA not extending into the CFA (iv) Unilateral EIA occlusion not involving the origins of internal iliac or CFA
C	(i) Bilateral CIA occlusions (ii) Bilateral EIA stenosis (3–10 cm long) not extending into the CFA (iii) Unilateral EIA stenosis extending into the CFA* (iv) Unilateral EIA occlusion that involves the origins of internal iliac and/or CFA* (v) Heavily calcified unilateral EIA occlusion with or without involvement of origins of internal iliac and/or CFA*
D	(i) Infrarenal aorta-iliac occlusion** (ii) Diffuse disease involving the aorta and both iliac arteries requiring treatment (iii) Diffuse multiple stenosis involving the unilateral CIA, EIA, and CFA* (iv) Unilateral occlusions of both CIA and EIA (v) Bilateral occlusions of EIA (vi) Iliac stenosis in patients with AAA requiring treatment and not amenable to endograft placement or other lesions requiring open aortic or iliac surgery**

**Endovascular treatment is not intended for these lesion types. *Lesions involving CFA with severe stenosis are excluded in these types. TASC II, Trans-Atlantic Inter-Society Consensus-II; CIA, common iliac artery; EIA, external iliac artery; CFA, common femoral artery; AAA, abdominal aortic aneurysm.

Table 3: Lesion and procedure characteristics.

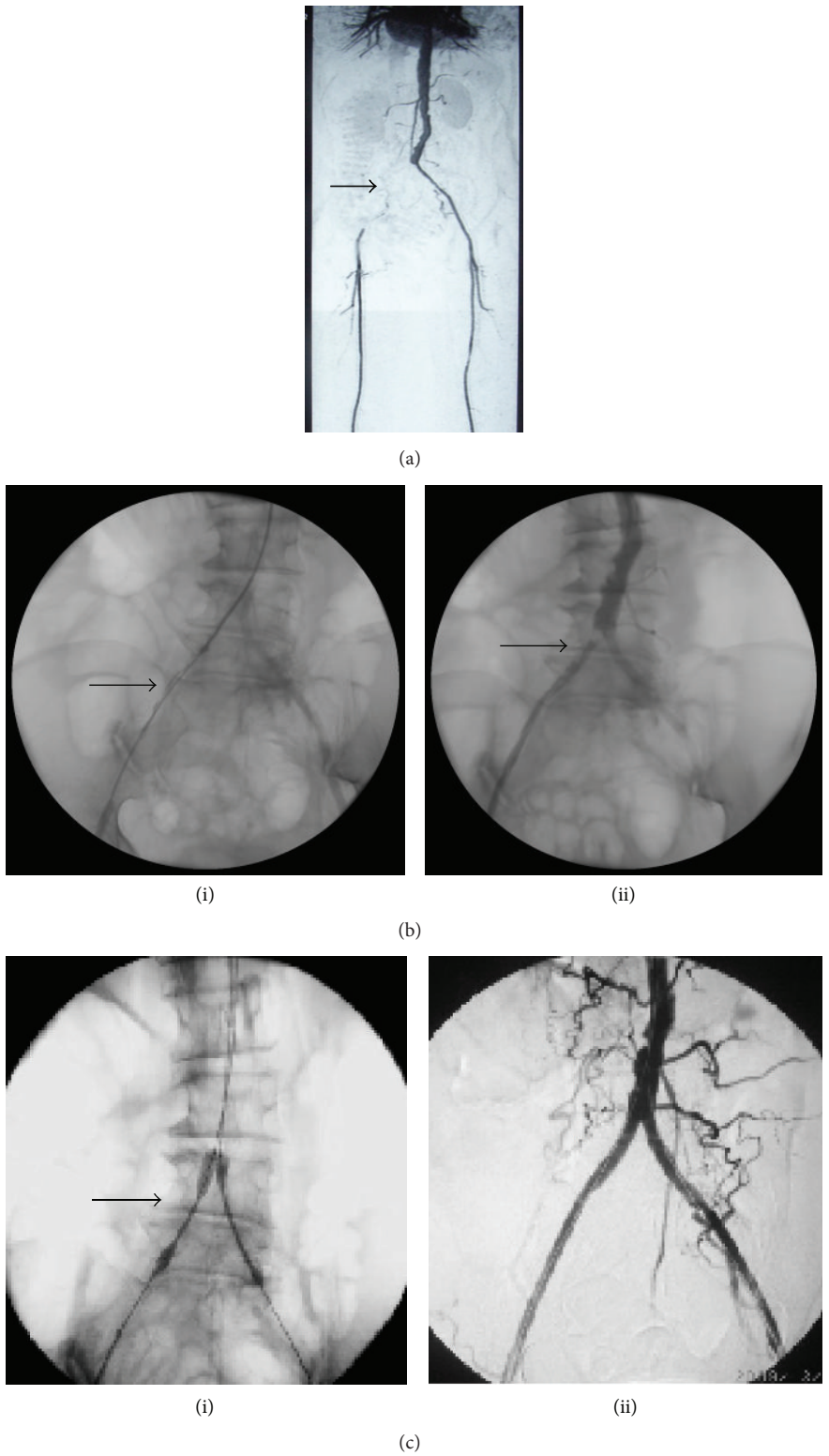
Characterization	Frequency (%)
Lesion type	
TASC II A	0 (00.0%)
TASC II B	25 (39.7%)
TASC II C	21 (33.3%)
TASC II D	17 (27.0%)
Number of stents implanted (per patient)	
5 stents	8 (12.7%)
3 stents	17 (27.0%)
2 stents	38 (60.3%)
Self-expanding stents	55 (34.2%)
Balloon-expanding stents	98 (60.9%)
Covered stents	8 (5.0%)
Catheter-directed thrombosis	8 (12.7%)

in aorta-bilateral iliac artery with stenosis in SFA, 12 patients had occlusions in bilateral common iliac artery, 9 patients had occlusions in left common iliac artery with stenosis at bilateral SFA, and 17 patients had occlusions in right common iliac artery. The patients were categorized according to the stratification of Trans-Atlantic Inter-Society Consensus-II (TASC II) [7]. Table 2 illustrated the standardised TASC II stratification. The number of patients in different TASC types and the number of implants in the patients were summarized in Table 3.

3.2. Short Term Assessment of Kissing-Stenting Treatment. Prior to operation, CDT with urokinase was used to improve perfusion. In all patients, Kissing-stents were placed successfully with restoration of the patency of aorta-iliac bifurcation segments and the technical success rate was 100%. In total, 161 stents were applied in bilateral common iliac arteries, which included 55 self-expanding stents, 98 balloon expandable stents, and 8 covered stents. In more detail, 5 stents were applied in 8 patients, 3 stents in 17 patients, and 2 stents in 38 patients. After CDT 3 patients originally presenting TASC II D improved to TASC II B, 3 patients improved from TASC II C to TASC II B, and 2 patients improved from TASC II D to TASC II A, separately.

Shortly after the surgery, the stents occluded in 2 patients due to thrombosis and thus they were treated with CDT immediately. Six months after the surgery, stent thrombosis occurred in 7 patients who did not take aspirin regularly after the initial intervention. In these patients, 3 had hematoma, 2 had stroke, 1 had worse renal function, and 1 had brachial pseudoaneurysm. CDT was repeated in these patients to alleviate the thrombosis. The overall complication rate was 14.3% (Table 4). No in-hospital mortality was recorded. Figure 1 demonstrated a female patient who was featured as severe right iliac artery occlusive lesion and thrombolysis by the catheter during the operation. Kissing-stenting treatment resulted in significant improvement flow in the lesion artery. Figure 2 displayed another representative treatment of a male PAD patient before and after Kissing-stenting angiography.

3.3. Long Term Assessment of Kissing-Stenting Treatment. All the patients were followed up to 7 years to evaluate the efficacy and safety of the stenting therapy. The ABI index increased 3.3-fold by Kissing-stenting treatment (0.28 ± 0.23 versus 0.75 ± 0.18; $P = 0.008$). All patients had complete alleviation



(a)

(i) (ii)

(b)

(i) (ii)

(c)

Figure 1: Case (1): stenting for a female PAD patient of 69 years. (a) CTA showed severe right iliac artery occlusive lesions about 12 cm (arrow). (b) (i) Thrombolysis by catheter (arrow). (b) (ii) Two days later, angiogram showed right iliac artery occlusive lesion changed from TASC II D to B (arrows). (c) (i) Endovascular angioplasty by Kissing-stenting (arrow). (c) (ii) Excellent result at right iliac artery.

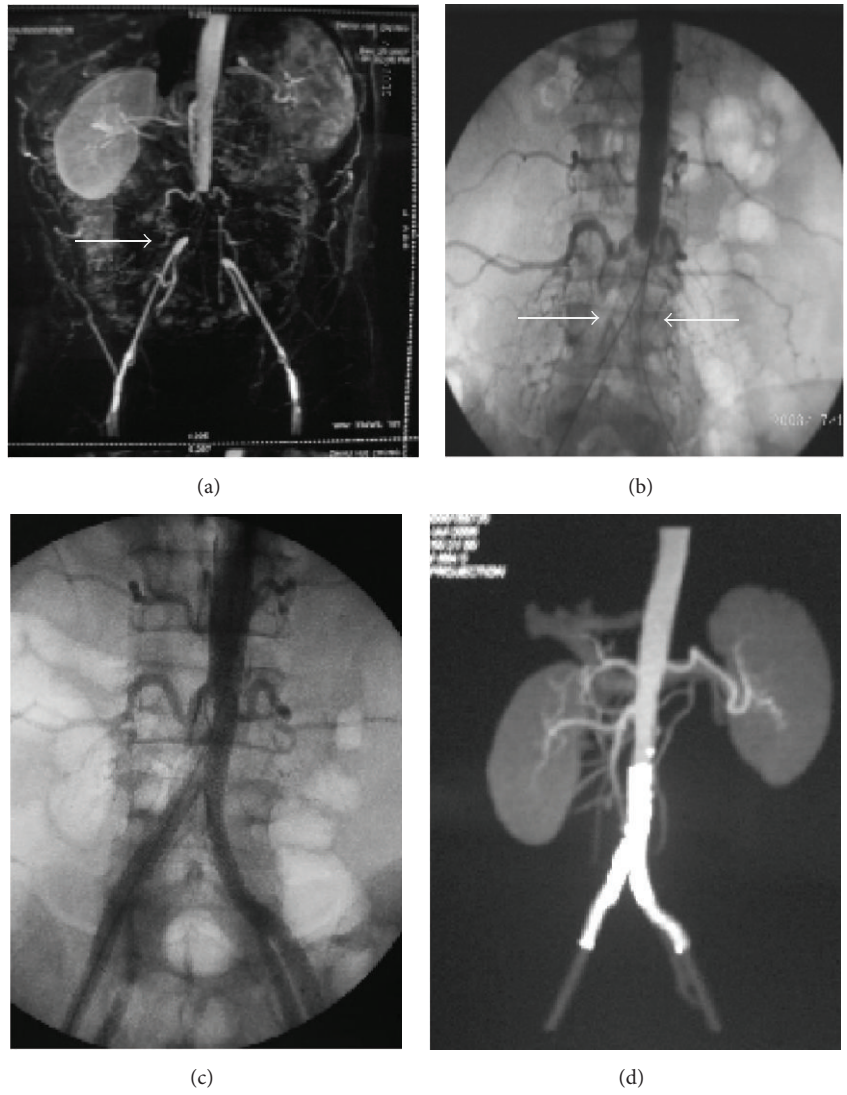


Figure 2: Stenting for a male PAD patient of 46 years. (a) CTA showed aorta-iliac artery occlusion lesions (arrow). (b) Two guide wires pass to aorta-iliac artery from bilateral femoral artery (arrows). (c) After dilation by balloon, Kissing-stents placement involving the distal aorta and bilateral common iliac is performed. (d) Excellent result.

Table 4: Frequency of complications.

Complications	Number (%)
Total	9 (14.3%)
In-stent thrombosis	2 (3.2%)
Hematoma	3 (4.8%)
Stroke	2 (3.2%)
Worsening renal function	1 (1.6%)
Brachial pseudoaneurysm	1 (1.6%)

of the claudication or rest pain symptoms, the foot ulcers cured, and the average hospitalization days were about 7 days. Overall, the primary patency rate was 87.3%, 77.4%, 71.1%, and 65.0%, and the second patency rate was 95.2%, 92.5%, 89.5%, and 85.0% at 1 year, 3 years, 5 years, and 7 years, respectively.

4. Discussion

In 1964, Dotter and Judkins first reported successful interventions in 9 patients who could not accept the traditional surgical operation and were subjected to limb amputation [8]. Nearly two decades later, Kissing-Technique has been used to treat peripheral ischemia, which includes Kissing balloon angioplasty and Kissing-stents [9]. Till present, a number of individual cases have demonstrated the efficacy of the stenting technique. In 2002, Haulon et al. reported 89.5% patency rate at 1 year and 79.4% patency rate at 3 years following stenting [10]. Likewise, Houston et al. illustrated that the primary patency rate was about 68% and the second patency rate went up to 86% after 10 years of Kissing-stenting [11]. Similarly, Björses et al. reported that aorta-iliac Kissing-stents were an alternative treatment to conventional surgery for TASC A–D lesions [12]. The procedure has low

mortality and morbidity with good patency at 3 years after stenting. Although Kissing-stenting is a promising tool for the treatment of PAD, more efforts are needed to obtain better long term patency rate. Nevertheless, the efficacy of Kissing-stenting in Chinese PAD patients was not well defined.

The main findings of this study are that Kissing-stenting success rate was 100% and ABI was 3.3-fold improved ($P < 0.01$) 7 years after stenting in the Chinese PAD patients. The primary patency rates were 87.3%, 77.4%, 71.1%, and 65.0%, respectively, after 1, 3, 5, and 7 years of stenting. The secondary patency rate reached 95.2%, 92.5%, 89.5%, and 85.0%, respectively, at 1, 3, 5, and 7 years following stenting. Thus, our data suggest that Kissing-stenting technique for aorta-iliac lesions is safe and effective for long-segment aorta-iliac occlusions. In the literature, Aihara et al. reported that the primary patency after 1 and 5 years of stents was 87% and 73%, respectively, in the treated PAD patients ($n = 190$) [13]. In the meanwhile, Pulli et al. reported that the primary patency rate after 5 years of stents was 82.4% and the secondary patency rate was 93.1% in the patients with iliac artery occlusion ($n = 109$) [14]. Taken together, our data indicate that the long term efficacy of Kissing-stents in the treatment of PAD patients could be comparable between Chinese and western countries.

During endovascular procedures, how to pass through the occlusive lesions was a crucial step in the past and got almost resolved now. However, embolism or occlusion in the contralateral common iliac artery may occur due to shedding of atherosclerotic plaques or thrombotic material during PTA or stenting in unilateral iliac artery in patients with no anchoring (landing zone) or a short stump (≤1 cm) in the aorta-iliac junction or proximal common iliac artery. Thirty days after stenting, reendothelisation in aorta appears in the place where the stents are implanted.

Hereby, we summarized our experience as follows: (1) balloons and stents should be selected in the same diameter with the same length and from the same manufacturer. The diameter of stenting could be oversized to 1.1-fold. (2) The Kissing-stents ought to be placed with the proximal ends in the aorta to the same level and then dilated simultaneously. (3) Self-expanding nitinol stents are the primary choice for patients with long and tortuous lesions (≥60 mm) without heavy calcification and postdilated to the normal vessel diameter. (4) Balloon expandable stents are applied to shorter lesions (≥60 mm) with or without calcification, sometimes to reinforce self-expandable stents if necessary. (5) Covered stents are suggested to treat patients with predicted high rupture risk or with heavy calcification or residual thrombus.

There are several limitations in this study: (1) this is a single-center study with 63 patients in total. Thus the number of study subjects is limited; (2) in the study, we did not categorize the PAD patients on the status of thrombosis. Therefore, it is not clear whether the different type of thrombolysis (acute, subacute, and chronic thrombosis) might have an impact on stenting treatment.

To date, thrombolytic therapy has been accepted as the optional initial treatment for acute limb ischemia but occasionally used to treat patients with chronic limb ischemia. Our experience is to apply CDT with urokinase from brachial access in patients with long-segment aorta-iliac occlusion (≥60 mm) to improve perfusion [15]. We injected urokinase 750,000 units per day for two days and continuous infusion of heparin (400–600 IU per hour) so that the activated partial thromboplastin time was maintained two times higher than normal range. We achieved a higher endovascular angioplasty success rate with no serious complications. Although catheter-directed thrombolysis cannot completely dissolve a clot, it may decrease the complexed surgical procedure and reduce hospitalization time and cost [16]. In addition, we believe that there may be coexistence of stable thrombus and unstable thrombus in the segments of occluded artery. CDT could help to decrease the unstable thrombus load before angioplasty and/or stenting. Therefore, it allows target-specific treatment and decreases the occurrence of embolization by removal of the relatively unstable thrombus components and improvement in the runoff status.

5. Conclusions

In conclusion, endovascular management for aorta-iliac stenosis and occlusive disease by Kissing-stenting technique is safe and effective and can raise the endovascular operation success rate with lower complications. Preoperative CDT with urokinase is effective for long-segment aorta-iliac occlusions (≥60 mm).

Conflict of Interests

The authors declare no association with any other individual or company having a vested interest in the subject matter/products and the stentings mentioned in this paper.

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Research Article

Endothelium-Independent Hypoxic Contraction Is Prevented Specifically by Nitroglycerin via Inhibition of Akt Kinase in Porcine Coronary Artery

Huixia Liu,[1,2] Yanjing Li,[1] Yuanming An,[1] Peixin He,[1] Liling Wu,[1,3] Yuansheng Gao,[1,3] and Dou Dou[1,3]

[1]*Department of Physiology and Pathophysiology, Peking University Health Science Center, 38 Xue Yuan Road, Beijing 100191, China*
[2]*Department of Physiology, Heze Medical College, Heze, Shandong, China*
[3]*Key Laboratory of Molecular Cardiovascular Science (Peking University), Ministry of Education, Beijing, China*

Correspondence should be addressed to Dou Dou; doudou@bjmu.edu.cn

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Objective. Hypoxia-induced sustained contraction of porcine coronary artery is endothelium-independent and mediated by PI3K/Akt/Rho kinase. Nitroglycerin (NTG) is a vasodilator used to treat angina pectoris and acute heart failure. The present study was to determine the role of NTG in hypoxia-induced endothelium-independent contraction and the underlying mechanism. *Methods and Results*. Organ chamber technique was used to measure the isometric vessel tension of isolated porcine coronary arteries. Protein levels of phosphorylated and total Akt were determined by western blot. A sustained contraction of porcine coronary arteries induced by hypoxia was significantly reduced by NTG but not by isoproterenol. This contraction was also inhibited by DETA NONOate, 8-Br-cGMP, which can be reversed by ODQ, and Rp-8-Br-PET-cGMPS. The restored contraction was blocked by LY294002. The reduction of Akt-p at Ser-473 by NTG, DETA NONOate, and 8-Br-cGMP was significantly inhibited by ODQ, PKG-I. The decrease in Akt-p level by NTG and 8-Br-cGMP was prevented by calyculin A but not by okadaic acid. *Conclusions*. These results demonstrated that the endothelium-independent sustained hypoxic vasoconstriction can be prevented by NTG and that the inhibition of PI3K/Akt signaling pathway may be involved.

1. Introduction

Since it was firstly reported by Vanhoutte in 1976 that acute hypoxia caused a rapid increase in tension of canine saphenous veins precontracted with acetylcholine [1], hypoxic vasoconstriction has been observed in a number of systemic vessel types including coronary arteries [2–9]. Subsequent studies demonstrate that the rapid hypoxic contraction is endothelium-dependent and closely associated with nitric oxide (NO) and soluble guanylyl cyclase (sGC) but unrelated to cGMP [4, 7, 8]. Our recent study revealed that cIMP, which was formed by sGC under hypoxic conditions, may play a role in the hypoxia-induced vasoconstriction [9]. This rapid endothelium-dependent hypoxic vasoconstriction is usually followed by a transient relaxation and then a sustained contraction in porcine coronary arteries when exposed to hypoxia. Our recent study suggests that this sustained hypoxic contraction is endothelium-independent and mediated by PI3K/Akt/Rho kinase [10].

Akt is a serine/threonine kinase which plays important roles in cell survival, differentiation, proliferation, metabolism, migration, and apoptosis [11–16]. It is increasingly recognized as being a pivotal modulator of vascular tone by regulating endothelial nitric oxide synthase, L-type calcium channel, Rho kinase, and phosphodiesterase type 5 [17–25]. Activation of Akt depends on the integrity of its pleckstrin homology (PH) domain, which is required for the membrane translocation of the kinase. After being translocated to the membrane via binding of phosphatidylinositol (3,4,5)-triphosphates, Akt is phosphorylated by its upstream kinases at Thr-308 and then at Ser-473 [26]. Akt is not fully activated until both Thr-308 and Ser-473 are

phosphorylated. Therefore, the full activation of Akt depends on the phosphorylation of Ser-473 [27]. Our recent study showed that the hypoxia-induced Akt phosphorylation at Ser-473 is associated with changes in the tension of porcine coronary arteries [10].

Nitroglycerin (NTG) is a commonly used medicine in the treatment of angina pectoris and acute heart failure. It causes vasodilatation after being converted into NO or a NO related intermediate in the cytoplasm, followed by cGMP elevation due to activation of sGC and PKG [28, 29]. Our recent study showed that Akt activity was inhibited by NO via inhibiting the phosphorylation at Ser-473 of the enzyme in porcine pulmonary artery [30]. The present study was intended to determine whether the endothelium-independent hypoxic contraction is prevented by NTG via inhibition of Akt in porcine coronary artery.

2. Materials and Methods

2.1. Reagents. The following drugs were used (unless otherwise specified, all were obtained from Sigma, St. Louis, MO, USA): 8-Br-cGMP (8-bromo-guanosine 3′5′-cyclic monophosphate), calyculin A, DETA NONOate[2,2′-(hydroxynitrosohydrazono) bis(ethanamine)], LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], nitroglycerin (NTG, Beijing Yimin Pharmaceutical Co., Ltd., Beijing, China), okadaic acid, Rp isomer (Rp-8-Br-PET-cGMPS; Biolog Life Science Institute, Bremen, Germany), and U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α).

LY294002, calyculin A, and okadaic acid were dissolved in DMSO (final concentration; <0.2%). Preliminary experiments showed that DMSO at the concentration used had no effect on contraction to U46619 and relaxation induced by nitroglycerin. The other drugs were prepared using distilled water.

2.2. Porcine Coronary Arteries Preparations. Fresh porcine hearts were collected from a local slaughterhouse. The left anterior descending coronary arteries were carefully dissected and cut into rings in ice-cold modified Krebs-Ringer bicarbonate buffer. The vessels were denuded of the endothelium mechanically by inserting the tips of a watchmaker's forceps into the lumen of the vessel and rolling the vessel back and forth on saline-saturated filter paper. Animal handling and study protocols were in accordance with US National Institutes of Health guidelines. They were reviewed and approved by Animal Care and Use Review Committees of Peking University Health Science Center [31].

2.3. Vessel Tension Study. Rings of porcine coronary arteries were repeatedly rinsed and suspended in organ chambers filled with 10 mL of the modified Krebs-Ringer bicarbonate solution maintained at 37 ± 0.1°C and aerated with 95% O_2-5% CO_2 (pH = 7.4). Two stirrups passed through the lumen of the vessel ring: one was anchored to the bottom of the organ chamber and the other was connected to a strain gauge. The isometric force was measured with ML785 PowerLab/8sp recording and Analysis System (ADInstruments Pty Ltd., Castle Hill, Australia) [32].

At the beginning of the experiment, each vessel was stretched to its optimal resting tension by stepwise stretching until the contraction of the vessel ring to 100 mM KCl reached a plateau. The optimal resting tension of porcine coronary arteries was ~2.5 g. Then one hour of equilibration was allowed. After equilibration certain inhibitors including ODQ, Rp-8-Br-PET-cGMPS, and LY294002 were added and remained throughout the experiment. In some experiments, NTG, isoproterenol, DETA NONOate, and/or 8-Br-cGMP were added 30 minutes before constriction. The effects of hypoxia (95% N_2-5% CO_2) were examined in vessels preconstricted with U46619 to a similar tension level. To eliminate the possible involvement of endogenous prostanoids and endothelium-derived nitric oxide, indomethacin (10^{-5} M, an inhibitor of cyclooxygenase) and nitro-L-arginine (10^{-4} M, an inhibitor of NO synthase) were administrated. All experiments were carried out in a parallel fashion.

2.4. Western Blot Study. Pretreatment of arterial rings: isolated porcine coronary arteries without endothelium were incubated in Krebs-Ringer bicarbonate buffer maintained at 37 ± 0.1°C and aerated with 95% O_2-5% CO_2 (pH = 7.4) in the presence of solvent, NTG, DETA NONOate, or 8-Br-cGMP. The inhibitors including ODQ, Rp-Br-PET-cGMPS, calyculin A, and okadaic acid were added at least 30 minutes before testing their effects.

Immunodetection: vessel rings were rapidly frozen with liquid nitrogen and homogenized in lysis buffer containing 34.67 mM SDS, 1 mM sodium orthovanadate, and 10 mM Tris base (pH 7.4). Tissue lysates each containing 20 μg of soluble protein were subjected to SDS-PAGE and electrotransferred to polyvinylidene fluoride membrane. Nonspecific binding of antibody was blocked by washing with TBS buffer containing 5% milk for 1 hour at room temperature. After two times brief washing with TBS plus 0.1% Tween-20 (TBS-T), the blot was incubated with the first antibody of Akt (Cell Signaling Technology, MA, USA; 1 : 1000 dilution) or Akt-p (S473) (Cell Signaling Technology, MA, USA; 1 : 1000 dilution) overnight at 4°C. Afterwards, the blot was washed three times with TBS-T buffer and then incubated with the secondary antibody for 1 hour at room temperature followed by another 3 times of washing and then developed using the chemiluminescent detection method (Amersham ECL). Proteins of Akt or Akt-p were quantified by densitometry using a Gel Doc 2000 densitometer (BIO-RAD, CA, USA) and normalized to scanning signals of beta-actin (Calbiochem, CA, USA).

2.5. Data Analysis. Data are shown as means ± SEM. Student's t-test for unpaired observations was used to compare the mean values of two groups. Mean values of more than two groups were compared using one-way ANOVA test, with the Student-Newman-Keuls test for *post hoc* testing of multiple comparisons. Statistical significance was accepted when the P value (two tailed) was less than 0.05. In all experiments, n represents the number of animals.

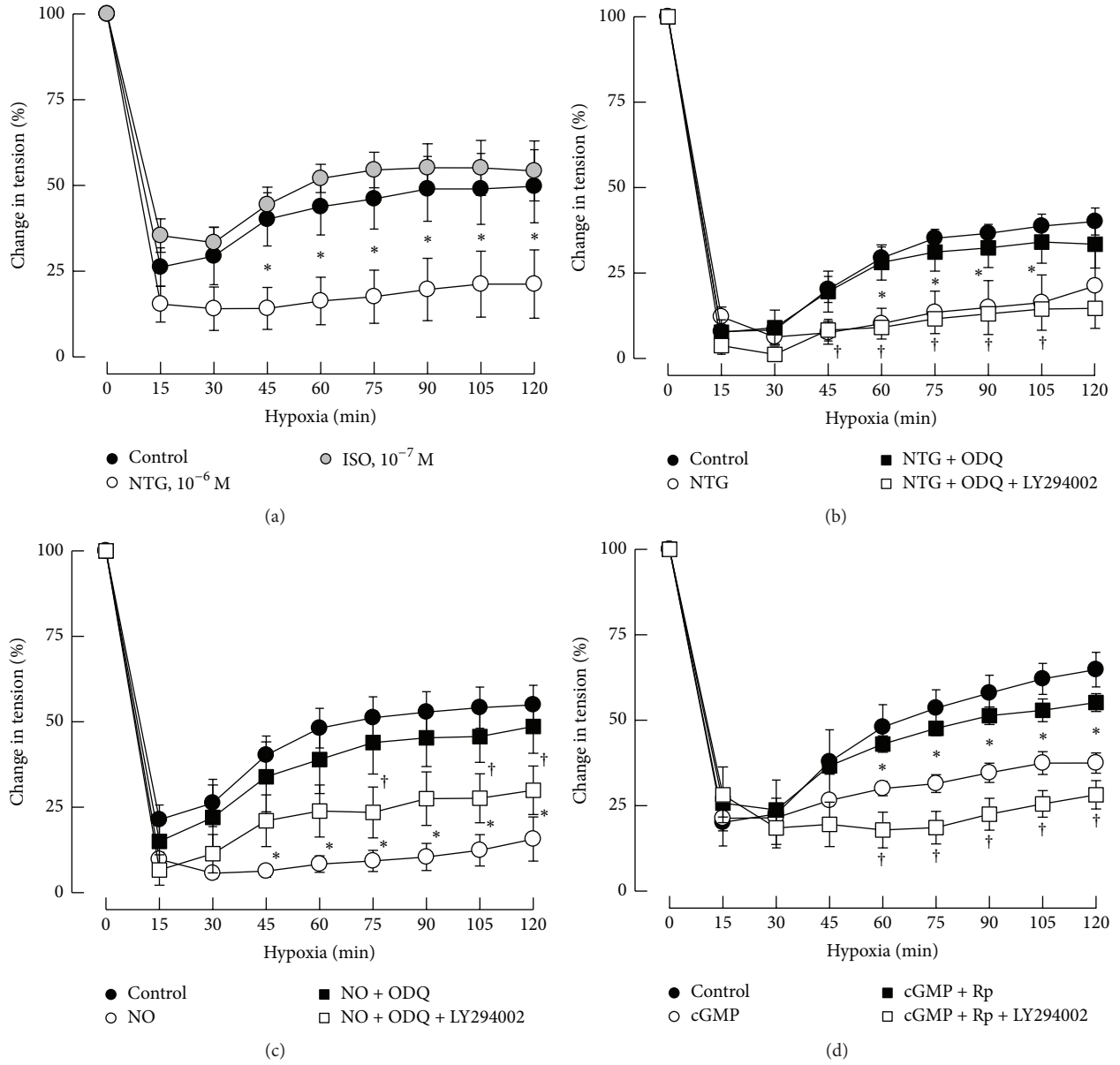

Figure 1: Effects of NTG, ISO, DETA NONOate, and cGMP on hypoxic vasoconstriction. The continuous contraction caused by hypoxia in porcine coronary arteries was prevented by NTG (10^{-6} M), but not by isoproterenol (ISO, 10^{-7} M; (a)). The prevention of the hypoxic vasoconstriction caused by NTG (10^{-6} M; (b)), DETA NONOate (NO, 10^{-4} M; (c)), and 8-Br-cGMP (cGMP, 10^{-4} M; (d)) was recovered by ODQ (3×10^{-5} M, a specific inhibitor of sGC; (b) and (c)) and Rp-Br-PET-cGMPS (PKG-I; 3×10^{-5} M; (d)). The restored hypoxic contraction of porcine coronary arteries by ODQ or Rp-Br-PET-cGMPS was reversed by LY294002 (10^{-5} M, a specific inhibitor of PI3K; (b), (c), and (d)). Data are means ± SEM; $n = 4$–6 for each group. *significantly different between the control group and treatment group with NTG, DETA NONOate, or 8-Br-cGMP ($P < 0.05$); †significantly different between the group with LY294002 and the group without LY294002 ($P < 0.05$).

3. Results

3.1. Hypoxia-Induced Sustained Vasoconstriction Was Prevented Specifically by NTG. Rings of porcine coronary arteries without endothelium were preconstricted with U46619 (3×10^{-7} M, a thromboxane A2 analog) before testing the response of hypoxia (95% N_2-5% CO_2). Hypoxia induced a rapid relaxation in the first 30 min, which was followed by a sustained contraction in porcine coronary arteries (Figure 1(a)). This sustained contraction of porcine coronary arteries was prevented by NTG (10^{-6} M; Figure 1(a)), but not by isoproterenol (ISO, 10^{-7} M; Figure 1(a)). Our preliminary experiment showed that isoproterenol (10^{-7} M) induced the same relaxation as NTG (10^{-6} M) in porcine coronary arteries preconstricted with U46619 (3×10^{-7} M, data not shown).

3.2. Involvement of NO/sGC/cGMP/PKG Pathway. The prevention of the hypoxic-induced vasoconstriction caused by NTG (10^{-6} M) could be recovered by ODQ (3×10^{-5} M),

a specific inhibitor of sGC (Figure 1(b)). Pretreatment of coronary arteries with NTG downstream molecule, NO donor (DETA NONOate, 10^{-5} M), or cGMP analog (8-Br-cGMP, 10^{-4} M) also attenuated the vasoconstriction caused by continuous hypoxia. These effects were blocked by ODQ or the inhibitor of PKG (PKG-I, Rp-8-Br-PET-cGMPS, 3×10^{-5} M). ODQ and PKG-I themselves had no effect on the hypoxic vasoconstriction (Figures 1(b), 1(c) and 1(d)).

3.3. Role of PI3K/Akt. The prevention of hypoxia-induced vasoconstriction of porcine coronary arteries caused by NTG, NO, or cGMP could be recovered by ODQ or PKG-I and the restored contractions were largely abolished by coincubation with LY294002, a specific inhibitor of PI3K (Figures 1(b), 1(c) and 1(d)).

3.4. Involvement of sGC in the Downregulation of Akt-p (S473) by NTG and NO. The protein levels of phosphorylated Akt at Ser-473 were significantly reduced by incubation with NTG (10^{-5} M) for 15 and 30 minutes (Figure 2(a)) or by incubation with DETA NONOate (10^{-4} M) for 1 to 30 minutes (Figure 2(c)). These effects were prevented by ODQ (3×10^{-5} M), a specific inhibitor of sGC (Figures 2(b) and 2(d)).

3.5. Involvement of PKG in the Downregulation of Akt-p (S473) by 8-Br-cGMP. The protein levels of phosphorylated Akt at Ser-473 were also significantly reduced by incubation with 8-Br-cGMP (10^{-4} M) for 45 to 90 minutes. The effect was blocked by Rp-8-Br-PET-cGMPS (3×10^{-5} M), a specific inhibitor of PKG (Figure 3).

3.6. Involvement of PP1 in the Downregulation of Akt-p (S473) by NTG and 8-Br-cGMP. Akt-p/Akt was decreased when treated with NTG (10^{-6} M; Figure 4(a)) for 15 min and 8-Br-cGMP (cGMP analog, 10^{-4} M; Figure 4(b)) for 60 min. Calyculin A (10^{-7} M, an inhibitor of PP1 and PP2A) but not okadaic acid (10^{-7} M, an inhibitor of PP2A) prevented the decrease in Akt-p (S473) caused by NTG and 8-Br-cGMP (Figure 4).

4. Discussion

Coronary artery spasm is a risk factor of acute ischemia heart disease such as angina pectoris and acute coronary syndrome [33, 34]. Endothelial dysfunction, hyperactivity of vascular smooth muscle cells, and other factors, including hypoxia, may be involved in the development of coronary vasospasm [2, 3, 34]. Recently, we found that prolonged hypoxia induced a transient initial contraction followed by a short term relaxation and a sustained contraction in porcine coronary arteries [10]. The first rapid hypoxic contraction is endothelium- or NO-dependent as reported by Vanhoutte and others [4, 9] while the second sustained contraction triggered by hypoxia is endothelium-independent [10]. Our study suggests that when oxygen content in the blood is decreased under certain disease conditions such as sleep apnea, high altitude sickness, and chronic obstructive pulmonary disease, the sustained hypoxic vasoconstriction may contribute to the development of spasm in coronary artery.

Both NTG and isoproterenol are vasodilators used in the treatment of cardiovascular disease. It is well known that vasodilatation caused by NTG is predominantly mediated by sGC/cGMP signaling pathway [28, 29] and the relaxation caused by isoproterenol involves adenylyl cyclase/cAMP signaling [35]. In our study, preincubation of nitroglycerin prevented the sustained vasoconstriction induced by hypoxia. By contrast, treatment of coronary arteries with isoproterenol had little effect. These results suggest that the endothelium-independent hypoxia-induced contraction is prevented specifically by NTG.

NTG causes vasodilatation via the intracellular conversion to NO or a NO related intermediate, which elevates cGMP by activating sGC [28, 29]. We found that the endothelium-independent hypoxic contraction was also prevented by NO. The suppression of hypoxic vasoconstriction by NTG or NO was recovered by ODQ, a specific inhibitor of sGC, suggesting that cGMP is involved in the effect of NTG and NO on hypoxia-induced vasoconstriction. PKG is one of the primary targets of cGMP. In our study the cGMP analog decreased the contraction induced by prolonged hypoxia in a manner sensitive to the inhibition of PKG by Rp-8-Br-PET-cGMPS. Thus, the activation of NO/sGC/cGMP/PKG pathway is involved.

Our previous work demonstrates that the activation of PI3K/Akt by hypoxia plays an important role in hypoxia-induced vasoconstriction [10]. This hypoxia-induced Akt phosphorylation at Ser-473 and vasoconstriction could be abolished by LY294002, an inhibitor of PI3K [10]. The present study shows that inhibition of PI3K/Akt pathway by LY294002 inhibited the effect of ODQ and Rp-8-Br-PET-cGMPS on the suppression of hypoxic vasoconstriction caused by NTG, NO, and 8-Br-cGMP. Hence, it appears that NTG prevents the hypoxia-induced coronary vasoconstriction mainly by inhibition of PI3K/Akt pathway.

Akt is a serine/threonine protein kinase involved in various cellular processes including the modulation of vascular smooth muscle responses [17]. In the present study the treatments of porcine coronary arteries with NTG, NO, or 8-Br-cGMP reduced the Akt phosphorylation at Ser-473, which was blocked by the inhibitors of sGC and PKG. It suggests that PKG activated by cGMP exerts an inhibitory effect on Akt by decreasing the phosphorylation of Akt at Ser-473, which is in line with our observation obtained in porcine pulmonary arteries [30].

PKG exerts its effects by phosphorylating the target proteins, including calcium activated potassium channels (BK channels) [36], IP_3R-associated PKG substrate [37], RhoA [38], and myosin phosphatase targeting subunit (MYPT1) [39]. The present study showed that Akt was dephosphorylated rather than phosphorylated by PKG, indicating that PKG might act on Akt indirectly. Indeed, some studies show that dephosphorylation of Akt is mediated by protein phosphatase 1 (PP1) or protein phosphatase 2A (PP2A) [40]. To determine whether PP1 and PP2A played a role in the dephosphorylation of Akt caused by PKG, the effects of calyculin A (CA), a nonselective inhibitor of PP1 and PP2A,

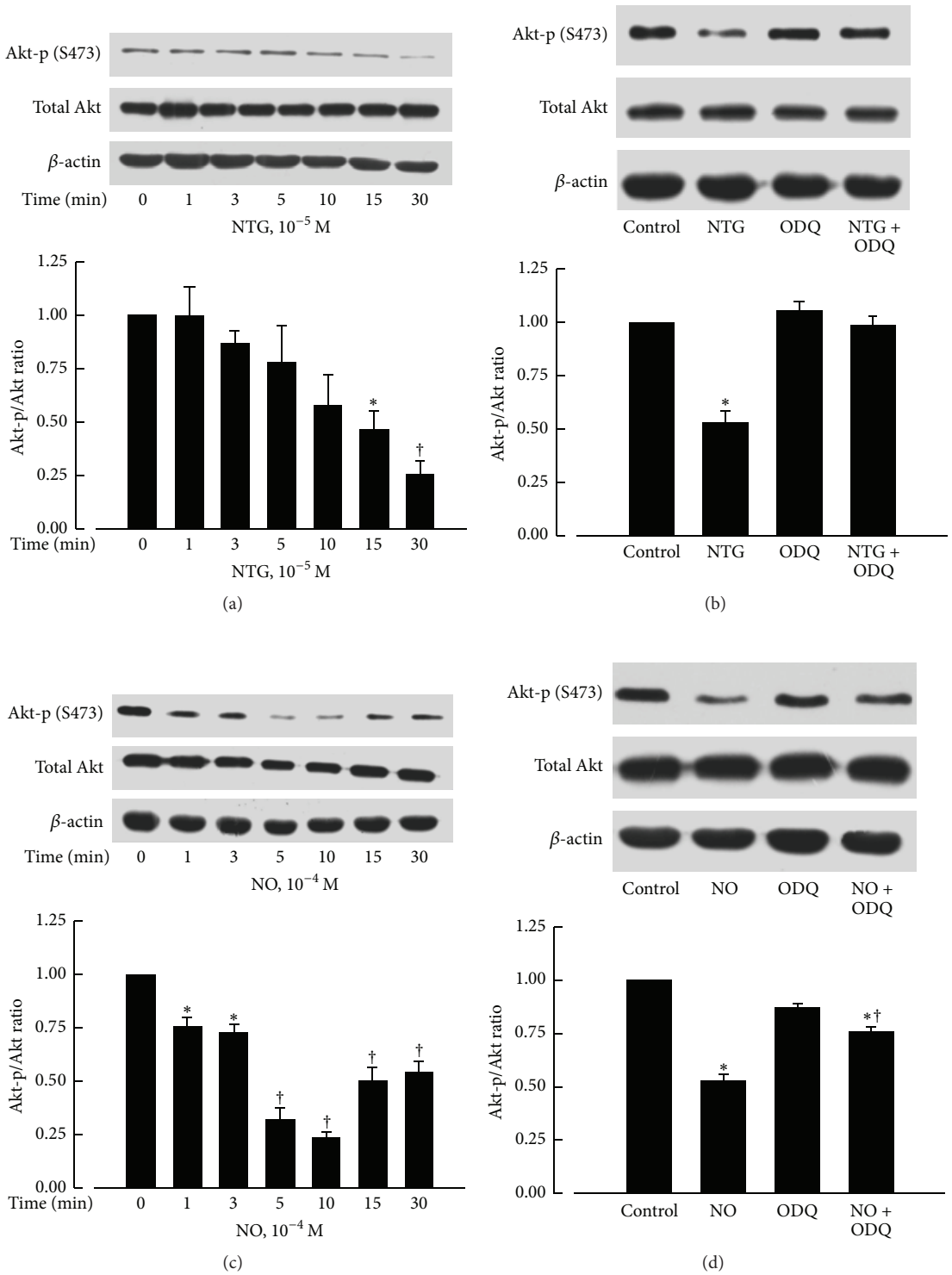

Figure 2: Effects of NTG and DETA NONOate on protein levels of Akt-p (S473) and total Akt. Akt-p (S473) was significantly decreased after NTG (10^{-5} M) treatment for 15 min (a) or DETA NONOate (NO, 10^{-4} M) treatment for 1 min (c), which could be recovered by ODQ (3×10^{-5} M; (b) and (d)). The upper panels are western blots. The lower panels are the summaries of densitometric scanning of proteins expressed as ratio of Akt-p (S473) to total Akt. Data shown as means ± SEM; $n = 6$–8 for each group. *significantly different from the control group ($P < 0.05$); †significantly different from the control group ($P < 0.01$, (a) and (c)); †significantly different from the NO group ($P < 0.05$, (d)).

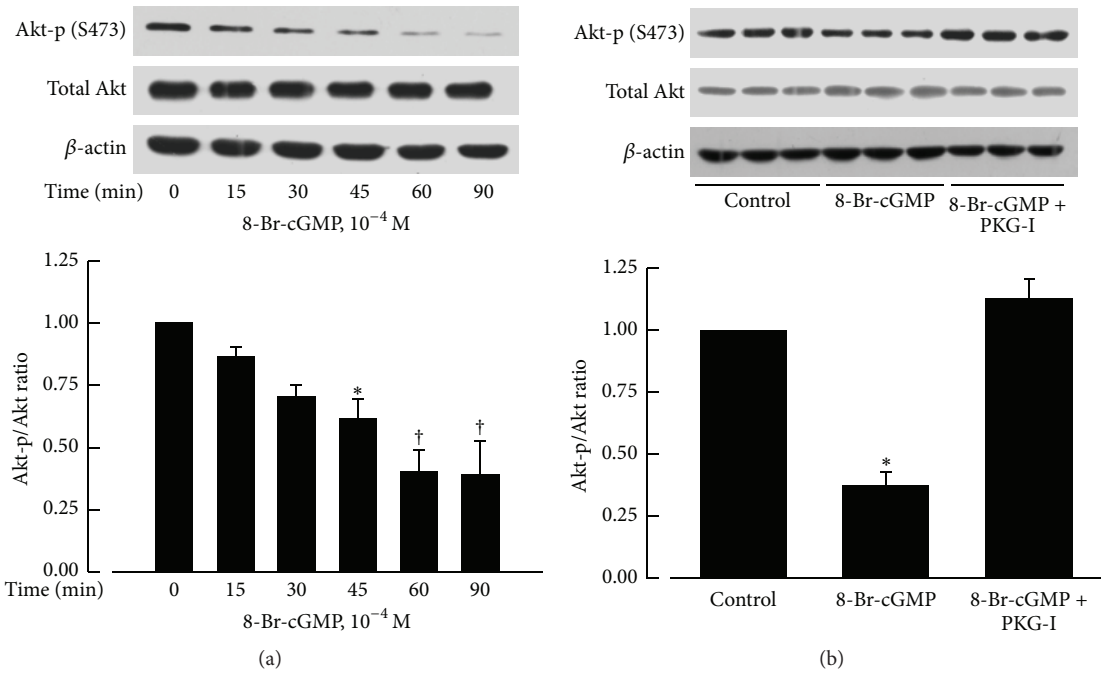

Figure 3: Effects of 8-Br-cGMP on protein levels of Akt-p (S473) and total Akt. Akt-p (S473) level was significantly reduced after 8-Br-cGMP (10^{-4} M) treatment for 45 min (a) and this effect could be prevented by Rp-8-Br-PET-cGMPS (PKG-I, 3×10^{-5} M; (b)). The upper panels are western blots. The lower panels are the summaries of densitometric scanning of proteins expressed as ratio of Akt-p (S473) to total Akt. Data shown as means ± SEM; $n = 6$ for each group. *significantly different from the control group ($P < 0.05$); †significantly different from the control group ($P < 0.01$).

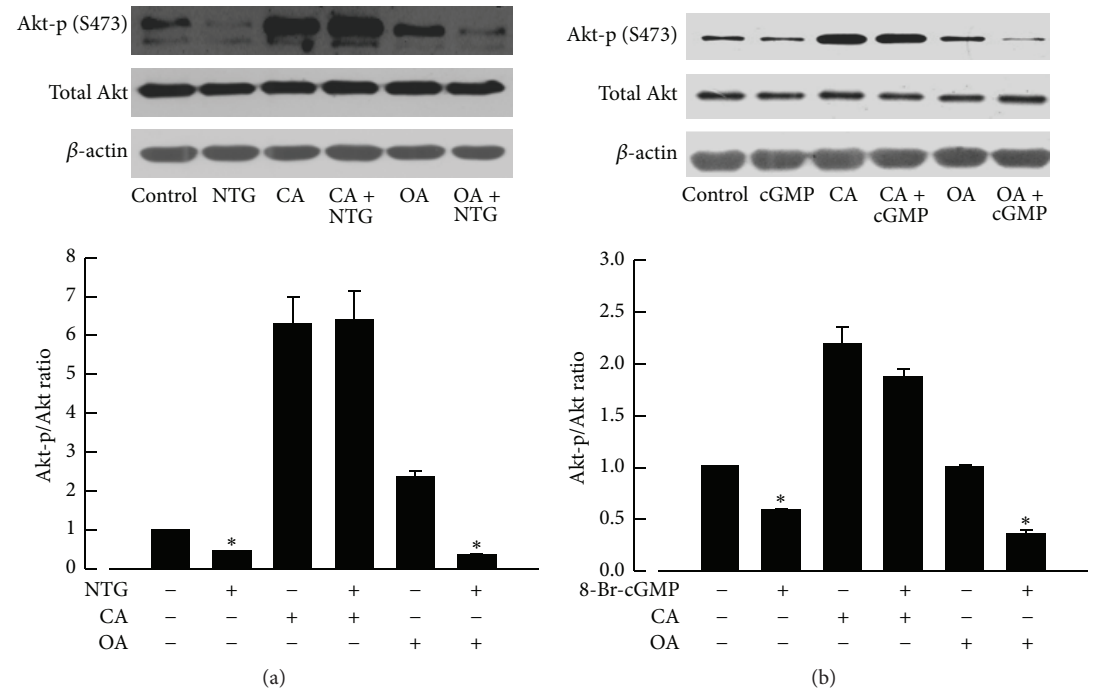

Figure 4: Effects of calyculin A and okadaic acid on the decrease in protein levels of Akt-p (S473) caused by NTG and 8-Br-cGMP. Akt-p (S473) was reduced when treated with NTG (10^{-6} M; (a)) for 15 min and 8-Br-cGMP (cGMP analog, 10^{-4} M; (b)) for 60 min. The reduction of Akt-p (S473) was prevented by calyculin A (CA, 10^{-7} M) but not by okadaic acid (OA, 10^{-7} M). The upper panels are western blots. The lower panels are the summaries of densitometric scanning of proteins expressed as ratio of Akt-p (S473) to total Akt. Data shown as means ± SEM; $n = 6$ for each group. *significantly different from the control group ($P < 0.05$).

and okadaic acid (OA), a selective inhibitor of PP2A, were studied [41, 42]. We found that the reduced phosphorylation of Akt induced by NTG and cGMP was reversed by CA but not by OA. Therefore, PKG may reduce the phosphorylation of Akt at Ser-473 mostly through PP1.

We herein demonstrate that pretreatment of NTG can prevent endothelium-independent vasoconstriction caused by prolonged hypoxia in porcine coronary arteries. The underlying mechanism may involve the activation of PKG induced by NTG, NO, or cGMP, which subsequently attenuates the phosphorylation of Akt at Ser-473 through PP1 in porcine coronary arteries, resulting in the inhibition of PI3K/Akt signaling pathway.

Conflict of Interests

All of the authors have no conflict of interests.

Authors' Contribution

Huixia Liu, Yanjing Li, and Yuanming An contributed equally to the research presented in this paper.

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Research Article

The Establishment and Characteristics of Rat Model of Atherosclerosis Induced by Hyperuricemia

Zhen Liu,[1,2,3] Tong Chen,[1,2] Haitao Niu,[4] Wei Ren,[1,2] Xinde Li,[1,2] Lingling Cui,[1,2] and Changgui Li[1,2]

[1] *Shandong Provincial Key Laboratory of Metabolic Disease, The Affiliated Hospital of Qingdao University, Qingdao 266003, China*
[2] *Shandong Gout Clinical Medical Center, Qingdao 266003, China*
[3] *Key Laboratory of Hypertension, Qingdao 266003, China*
[4] *Department of Urology, The Affiliated Hospital of Qingdao University, Qingdao 266003, China*

Correspondence should be addressed to Lingling Cui; cuillqd@163.com and Changgui Li; lichanggui@medmail.com.cn

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Epidemiological studies have identified hyperuricemia as an independent risk factor for cardiovascular disease. However, the mechanism whereby hyperuricemia causes atherosclerosis remains unclear. The objective of the study was to establish a new rat model of hyperuricemia-induced atherosclerosis. Wistar-Kyoto rats were randomly allocated to either a normal diet (ND), high-fat diet (HFD), or high-adenine diet (HAD), followed by sacrifice 4, 8, or 12 weeks later. Serum uric acid and lipid levels were analyzed, pathologic changes in the aorta were observed by hematoxylin and eosin staining, and mRNA expression was evaluated by quantitative real-time polymerase chain reaction. Serum uric acid and TC were significantly increased in the HAD group at 4 weeks compared with the ND group, but there was no significant difference in serum uric acid between the ND and HFD groups. Aorta calcification occurred earlier and was more severe in the HAD group, compared with the HFD group. Proliferating cell nuclear antigen, monocyte chemotactic factor-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 mRNA levels were increased in the HFD and HAD groups compared with the ND group. This new animal model will be a useful tool for investigating the mechanisms responsible for hyperuricemia-induced atherosclerosis.

1. Introduction

Uric acid is an end product of purine metabolism in humans and is excreted in the urine. Loss of uricase function means that humans and other primates have relatively higher levels of serum uric acid compared with rodents, providing the biochemical bases for the inflammatory response in gout and an increased risk of cardiovascular disease. Gertler et al. [1] initially proposed the existence of a complex interaction between uric acid and coronary heart disease in 1951; since then increasing numbers of studies have confirmed a link between raised serum uric acid levels and cardiovascular events. A recent meta-analysis of prospective studies showed that each additional 1 mg/dL of serum uric acid equated to a 12% increase in mortality for patients with coronary heart disease [2]. Raised serum uric acid levels are associated with approximately 70% increase in the risk of coronary heart disease. In addition to its direct cardiovascular effects, hyperuricemia has also been associated with increased risks for the development of hypertension, renal damage, and metabolic syndrome, which indirectly lead to the occurrence of cardiovascular events or affect the prognosis and therapy of cardiovascular disease.

However, conversely, some researchers suggest that the lack of uricase is an evolutionary advantage for primates [3, 4]. Hyperuricemia could help to stabilize blood pressure, and uric acid has antioxidant activities [3, 5, 6]. Hink et al. [7] reported that uric acid could also prevent the degradation of extracellular superoxide dismutase 3, which is a key enzyme for maintaining the functions of endothelial cells and blood vessels. Increased serum uric acid in patients with cardiovascular disease may be an important compensatory

Table 1: Sequences of primers.

Gene name	Product size	Sense primer	Antisense primer
Rat MCP-1	120 bp	TGTCCCAAAGAAGCTGTAGTATTTGT	TTCTGATCTCACTTGGTTCTGGTC
Rat ICAM-1	103 bp	GGTGGGC AAGAACCTCATCCT	CTGGCGGCT CAGTGTCTCATT
Rat VCAM-1	110 bp	CGAAAG GCCCAGTTG AAG GA	GAGCACGAGAAGCTCAGGAGA AA
Rat β-actin	120 bp	TGG ACA TCC GCA AAG AC	GAA AGG GTG TAA CGC AACTA

mechanism for oxidative stress during the course of the disease [8]. Hyperuricemia was associated with better prognoses in patients with stroke or other neurological disorders [9]. However, these observations fail to explain why higher uric acid levels are associated with a poorer prognosis in patients with cardiovascular disease. The validity of hyperuricemia as an independent risk factor for cardiovascular disease thus remains controversial.

In this study, we established an animal model to investigate the association between hyperuricemia and atherosclerosis risk and also examined the specific molecules involved in this process. The results have important implications for future clinical treatment strategies and for the early prevention of hyperuricemia and atherosclerosis.

2. Materials and Methods

2.1. Animal Model. Ninety male Wistar-Kyoto rats were purchased from the Animal Center of Beijing University, Beijing, China. Animal experiments were performed in accordance with the guidelines for the Principles of Laboratory Animal Care and the Guide for Care and Use of Laboratory Animals. Rats (200–220 g) were randomly divided into three groups fed a normal diet (ND; $n = 30$), high-fat diet (HFD; $n = 30$), or high-adenine diet (HAD; $n = 30$), respectively. HFD rats were administered intragastric (i.g.) vitamin D_3 (60 IU/kg) for 3 days followed by a dose of 5 mL/kg high-fat emulsion containing pyrimidine (200 g pork, 200 g cholesterol, 20 g bile salts, and 10 g propylthiouracil, dissolved in 1 L distilled water) twice daily, by intragastric administration. HAD rats were fed with fodder containing 10% yeast powder and administered adenine (50 mg/kg, i.g.) and potassium oxonate (100 mg/kg) subcutaneously twice a day (8 am and 4 pm). ND rats were administered an equal volume of normal saline i.g. and fed a normal diet. The rats were housed individually in specific pathogen-free conditions at a constant temperature (20–22°C) and humidity (45–55%) with a 12 h light-dark cycle. Rats from the three groups were sacrificed after 4, 8, and 12 weeks, respectively.

2.2. Serum Uric Acid and Lipid Measurements. At the end of the experimental periods, rats were fasted for at least 8 h and then anesthetized with 10% chloral hydrate. Blood samples were obtained from the right carotid artery after 4 h at room temperature and centrifuged at 3500 rpm for 15 min at room temperature. Serum was separated and uric acid and lipid levels were determined using an autoanalyzer (Toshiba, Japan).

2.3. Tissue Processing, Histology, and Immunohistochemistry. At the end of the experiment, the thoracic aorta (from the arch to the diaphragm) was harvested, cut in half, and either fixed in buffered formalin or snap frozen. Aorta rings were embedded in paraffin and sections were cut at 4 μm and prepared for hematoxylin and eosin (HE) staining. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was performed using an anti-PCNA primary antibody (1 : 500) and a horseradish peroxidase-conjugated goat anti-rat Ig secondary antibody (1 : 100) (Invitrogen, Basel, Switzerland).

2.4. Quantitative Real-Time Polymerase Chain Reaction. Total aorta RNA was extracted using Trizol reagent (Invitrogen). For each sample, cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara, China) according to the manufacturer's instructions. SYBR Green-based polymerase chain reaction (PCR) was performed in an automated thermal cycler (Bio-Rad) in a final volume of 25 μL, containing 2 μL cDNA solution, 12.5 μL of SYBR Premix Ex Taq (Takara), 1 μL of each primer (10 μmol/L), and 8.5 μL of ddH_2O. The cycling reaction was performed according to the manufacturer's instructions via a standard two-step PCR. Experimental Ct values were normalized to β-actin and relative mRNA expression was calculated in comparison with a reference sample. Each sample was run and analyzed in triplicate. The structures of the primers used are listed in Table 1.

2.5. Statistical Analyses. All data are expressed as mean ± standard deviation and were analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test as appropriate (GraphPad Prism version 5 software). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in Serum Uric Acid and Lipid Levels. Serum uric acid and lipid levels were measured in all groups. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were elevated in the HFD group compared with the ND group after 4, 8, and 12 weeks, respectively, while levels of UA were unchanged. However, serum levels of UA, TC, and HDL-C were increased in the HAD rats. Interestingly, TC levels were lower in the HAD compared with the HFD group (Figure 1). Furthermore, mortality was increased by 30% in the HAD group after 8 weeks, compared with the ND and HAD groups.

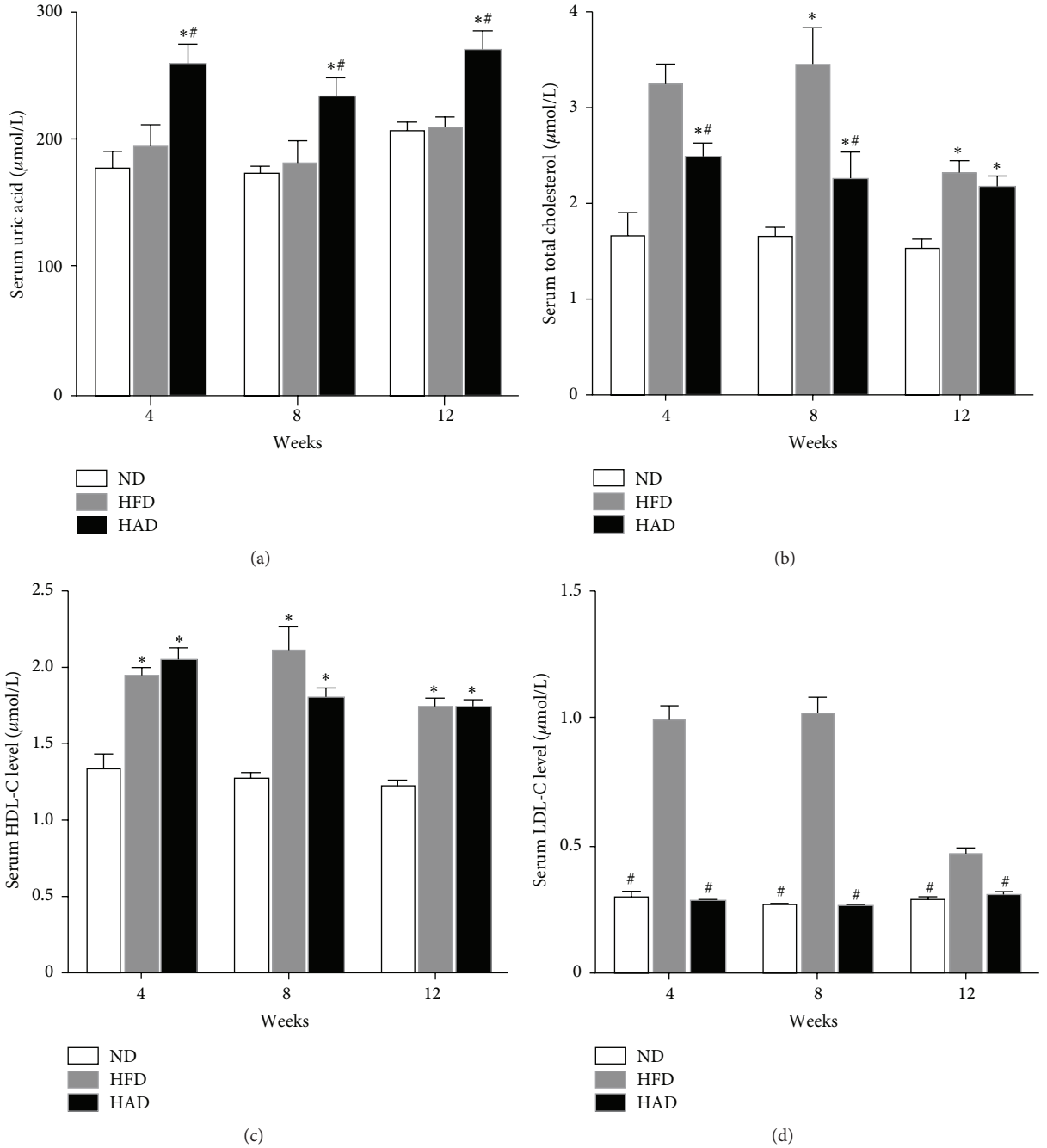

Figure 1: Serum uric acid and lipid levels in rats. Serum uric acid and lipid levels were analyzed using an autoanalyzer. (a) Serum uric acid, (b) serum TC, (c) serum HDL-C, and (d) serum LDL-C ($n = 8$–10). $^{*}P < 0.05$, compared with the ND group; $^{\#}P < 0.05$, compared with the HFD group.

3.2. Histopathological Changes in Aortas. There were no pathologic changes in the aortas of ND rats during the course of the experiment. However, foam cells were observed following mononuclear cell infiltration in the aortas of HFD and HAD rats after 4 weeks of treatment. After 8 weeks, numerous foam cells were formed and nuclear condensation appeared in medial smooth muscle cells in HFD rats, while calcium deposits were found in the aortas in HAD rats at 8 weeks and were more severe at 12 weeks. However, no calcium deposits were detected in the aortas of HFD rats up to 12 weeks. Hyaline degeneration occurred in both HFD and HAD rats after 12 weeks' treatment (Figure 2).

3.3. PCNA Expression in Aortas. PCNA expression in the aorta was examined by immunohistochemistry. Staining was primarily located in the medial smooth muscle cells (Figure 3) and was significantly increased in HFD compared with HAD rats (26.3 ± 1.9 versus 31.4 ± 1.4, $P < 0.05$).

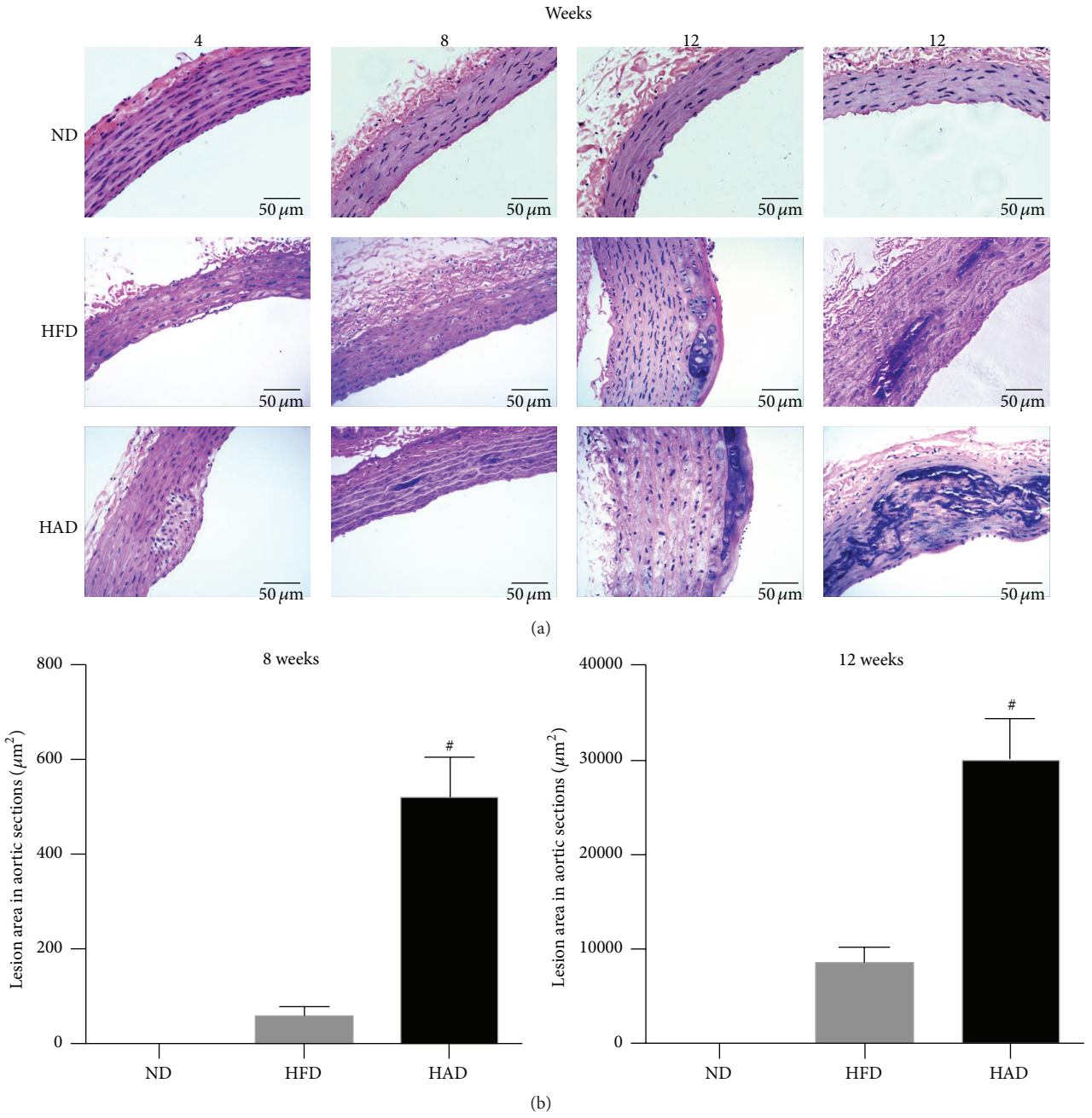

Figure 2: Histopathological examination of aorta in rats. All rats were sacrificed at 4, 8, or 12 weeks, respectively. (a) Aorta rings were stained with HE (×400 magnification). (b) Quantitation of lesioned area in HE-stained aorta sections using Image-Pro Plus software ($n = 6$). $^{\#}P < 0.05$, compared with the HFD group.

3.4. Monocyte Chemotactic Factor-1, Intercellular Adhesion Molecule-1, and Vascular Cell Adhesion Molecule-1 mRNA Expression in Aortas. Atherosclerosis in HAD rats was characterized by marked increases in aortic monocyte chemotactic factor-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) mRNA compared with the ND group, according to real-time PCR. However, upregulation of MCP-1 was lower in the HAD than in the HFD group.

4. Discussion

Hyperuricemia (defined as ≥7.0 mg/dL in male individuals and ≥6.0 mg/dL in female individuals) is caused by increased synthesis and/or reduced excretion of uric acid. The regulation of uric acid is complex, and hyperuricemia is also related to the development of multiple complications, including gout, hyperlipidemia, hypertension, and cardiovascular disease [10–13]. In the current study, we aimed to establish an animal

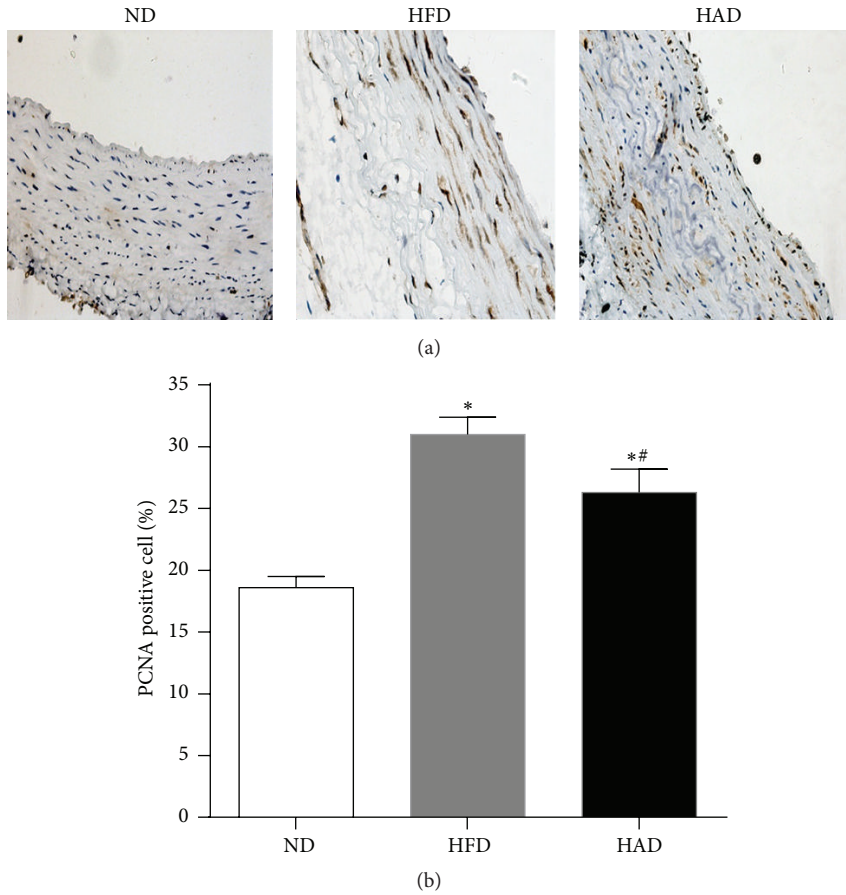

Figure 3: Immunohistochemical staining of aorta in rats. (a) Immunohistochemical staining for PCNA in the aorta (×400 magnification). (b) PCNA protein levels were quantified in each group ($n = 6$). $^{*}P < 0.05$, compared with the ND group; $^{\#}P < 0.05$, compared with the HFD group.

model of hyperuricemia-induced atherosclerosis by feeding rats HAD. This resulted in significant increases in uric acid, TC, inflammation, and calcification, which are known to be associated with atherosclerosis, suggesting that this model may help to further our understanding of the mechanisms whereby hyperuricemia induces atherosclerosis.

Rodents possess uricase, which metabolizes uric acid, and serum uric acid levels are therefore low. Further investigation of the relationship between hyperuricemia and atherosclerosis thus requires a hyperuricemic animal model. We created a rat model by feeding with a mixed diet of adenine, yeast power, and potassium oxonate. After 4 weeks, HAD rats had high serum TC levels. Dyslipidemia is important biochemical basis of atherosclerosis, and lipid accumulation both within and outside cells is a pathological feature of atherosclerotic plaques. Our results suggest that uric acid may interfere with lipid metabolism in rats resulting in abnormal lipid levels, which may provide a mechanism for hyperuricemia-induced atherosclerosis. HE staining showed that vascular smooth muscle cells (VSMCs) were damaged and ultimately disappeared in HAD rats after 4 weeks, with the appearance of foam cells. Furthermore, a high uric acid diet for 8 weeks resulted in diffuse deposits of calcium salts in the medial layer of the vasculature, damage to and fibrosis of subintimal smooth muscle cells, hyaline degeneration, and the appearance of foam cells. Notably, pathologic changes characteristic of atherosclerosis appeared sooner and became more severe in HAD compared with HFD rats.

VSMCs are one of the major cell types involved in atherosclerotic-plaque formation, and excessive proliferation of VSMCs is an important pathological characteristic of atherosclerosis. Soluble uric acid has been shown to cause proliferation of VSMCs [14]. During the early stage of atherosclerosis, medial smooth muscle cells undergo excessive proliferation and migrate to the intima, resulting in thickening of the intima and narrowing of the lumen. Transformation, proliferation, and migration of VSMCs are the basic pathological processes responsible for luminal stenosis [15, 16]. VSMCs are also important source of cytokines and play major role in maintaining and amplifying inflammatory proliferation. The number of PCNA-positive cells can thus reflect the status of cell proliferation [16] and provides important indicator for judging the biological activity and growth status of cells. In the current study, the surface area and thickness of the vessels (data not shown) and the ratio of PCNA-positive cells increased significantly in high uric acid

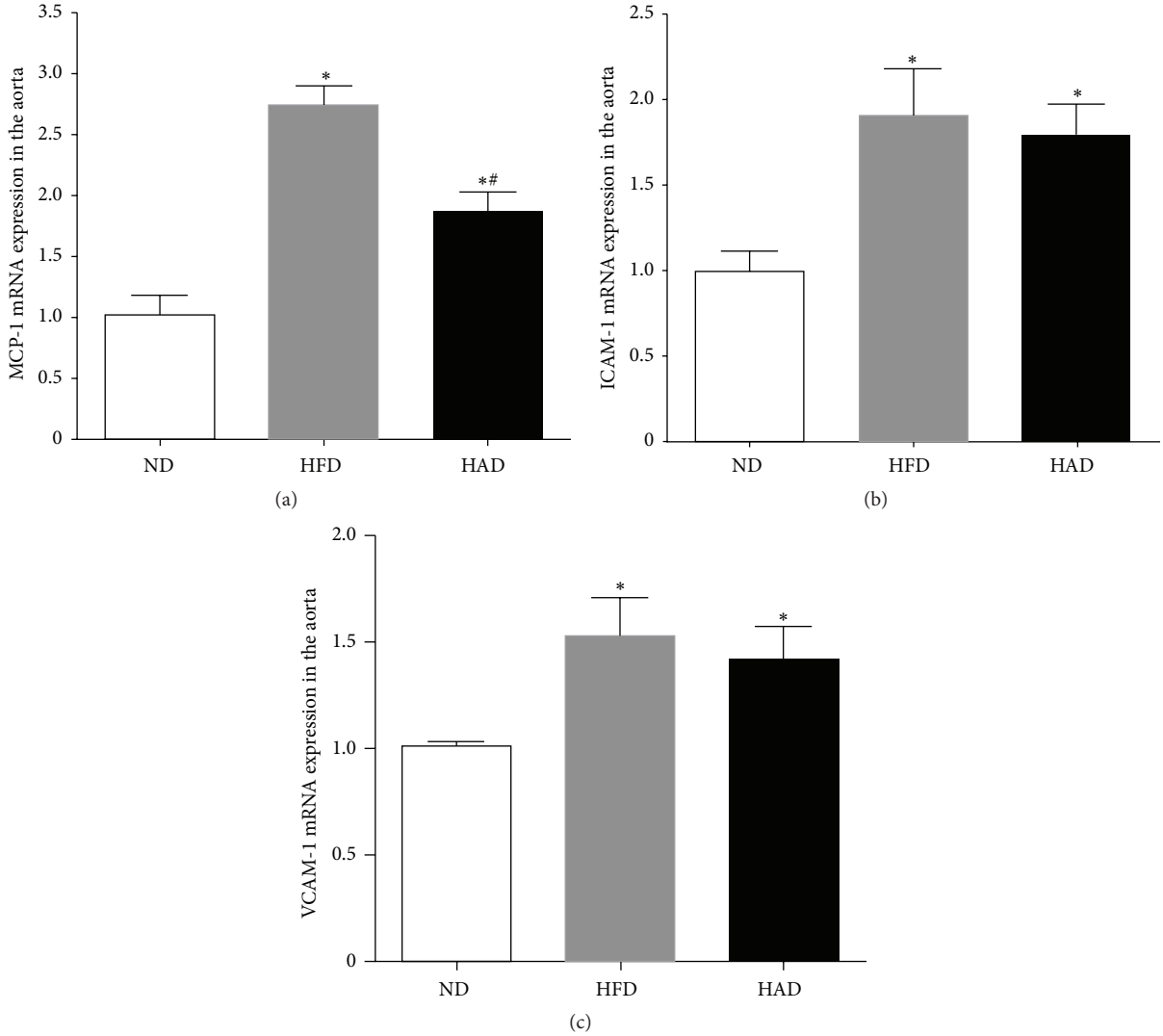

Figure 4: Gene expression in aorta of rats. Gene expression was measured by real-time PCR in each group. (a) MCP-1 mRNA, (b) ICAM-1 mRNA, and (c) VCAM-1 mRNA ($n = 8$–10). $^{*}P < 0.05$, compared with the ND group; $^{\#}P < 0.05$, compared with the HFD group.

rats. This result further confirmed the role of high uric acid levels in the proliferation of smooth muscle cells, which in turn mediate medial vascular sclerosis and the development of atherosclerosis.

We investigated the possible mechanisms of hyperuricemia-induced atherosclerosis in these model rats. Inflammation represents important mechanism for atherosclerosis, and uric acid is known to have a proinflammatory effect on vascular cells. In smooth muscle cells, high levels of uric acid induced MCP-1 by activating the transcription factor nuclear factor κ-B, mitogen-activated protein kinases, and cyclooxygenase 2, while expression levels of ICAM-1, VCAM-1, P-selectin, and E-selectin were also increased. We demonstrated that the mRNA expression levels of MCP-1, ICAM-1, and VCAM-1 in the aorta were increased in HAD rats compared with control rats, based on reverse-transcription PCR (Figure 4). Only upregulation of MCP-1 was lower in the HAD compared with the HFD group. These results indicate that high uric acid levels caused vascular damage and the development of atherosclerosis through upregulation of MCP-1, ICAM-1, and VCAM-1 mRNAs.

5. Conclusions

We established an animal model of hyperuricemia-induced atherosclerosis and found no significant difference between hyperuricemia and hypercholesterolemia in causing atherosclerosis in rats. High uric acid levels may cause atherosclerosis via disturbing lipid metabolism, promoting the proliferation of VSMCs, and by activation of inflammation. Hyperuricemia should therefore be regarded as an important risk factor for the occurrence and development of atherosclerosis, and the treatment of hyperuricemia represents an important approach to its prevention and treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zhen Liu, Tong Chen, and Haitao Niu contributed equally to this work. All authors approved the final paper as submitted and agree to be accountable for all aspects of the work.

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