Wnt3a promoted the therapeutic of angiogenesis on lower leg ischemia with endothelial progenitor cells

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Abstract: In our previous research, EPCs has been isolated from animal marrow cells and cord blood and improved the angiogenesis performance. Here we explored the effect of over-expression of Wnt3a gene in endothelial progenitor cells (EPCs) and demonstrated a new therapeutic angiogenesis strategy using genetically modified EPCs. As we previously reported, EPCs could be cultivated from animal peripheral blood. The adenovirus vector encoding Wnt3a gene was transfected into EPCs. Overexpression of Wnt3a firstly activated the Wnt/β-catenin signaling pathway led to increased nuclear translocation of β-catenin and increased secretion of angiogenic cytokines (vascular endothelial growth factor-a). It enhanced the survival, proliferation, and differentiation of EPCs. What’s more, combination of transplantation of EPCs with injection of adenovirus vector encoding Wnt3a gene into the hind limb ischemic model of mouse significantly improved blood flow and tissue capillary density compared with EPCs only treatment group and hind limb ischemic model. Over expression of Wnt3a in the EPCs augmented the in vitro and in vivo angiogenic potency of these cell populations. These data provide evidence that the Wnt3 pathway potentially has a key role in the angiogenic properties of EPCs. Furthermore, the genetic modification of EPCs to stimulate this signaling pathway would promote the efficacy of the therapeutic angiogenesis.

Keywords: Lower leg ischemia, peripheral arterial disease, EPCs, therapeutic angiogenesis, Wnt/β-catenin signaling pathway

Introduction

The lower leg ischemia, occurs when an artery in the legs is partially or completely blocked by plaque, suppressing blood flow and reducing the amount of oxygen supplied to the heart. Leg ischemia is the most severe form of peripheral arterial disease, which commonly affects the arteries supplying the leg and is mostly caused by atherosclerosis and is common in old age, putting patients at risk for heart attack and limb loss[1-4]. Restriction of blood flow due to arterial stenosis or occlusion often leads patients to intermittent claudication. Any further reduction in blood flow causes ischemic pain even while resting, which affects the foot. Ulceration and gangrene may then supervene and can result in loss of the legs or feet that fail to heal. Furthermore, based on an analysis of National Health and Nutrition Examination Survey (NHANES) 1999-2002 data it was showed that the prevalence of lower extremity disease increased significantly with age: from 12.3% in those aged 40 to 59 to 26.2% in those aged 60 to 74 and 40.8% in those aged 75 and older [5].

The treatment options include injection of an anticoagulant, thrombolysis, embolectomy, surgical revascularization, or amputation [6]. Our clinical practice showed that medication can only partly reduce clinical symptoms; although surgical revascularization benefits ischemia reperfusion, there is still postoperative complications/mortality; specifically, a great deal of patients missed operation timing due to poor blood flow in peripheral vessel. For example, about 150 to 200 per million of the population progress to critical limb ischemia each year,
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although a great deal of patients can undergo revascularization, which has a reasonable chance of saving the limb, (recently the Vascular Surgical Society found a success rate of over 70% for these patients), many patients still require major amputation [7]. Besides, it is difficult and costly for rehabilitation of elderly patients after amputation. Thus, there is yet a long way to go for significantly improving ischemia reperfusion as well as exploring effective treatments to markedly reduce the disability rate.

Previous studies [8] have identified that there are small amount of isolated endothelial progenitor cells (EPC) from human peripheral blood, in response to cytokine stimulation, these cells are mobilized from bone marrow and home to the ischemic tissue and contribute to new vessel formation [9, 10]. Based on the potential role of augmenting collateral vessel growth to ischemic tissues, EPCs have been investigated as an agent for therapeutic angiogenesis [11-17]. These studies have shown that administration of EPCs to animals with limb ischemia can enhance neovascularization and salvage tissue. Human clinical trials using autologous progenitor cells also showed improved blood flow [18].

In our previous research, EPCs have been isolated from animal marrow cells and cord blood and improved the angiogenesis performance [19, 20].

However, several limitations still exist in the therapeutic application of EPCs. Firstly, the amount of available EPCs needed for therapeutic angiogenesis is limited compared to the great amounts of peripheral blood that are required [21]. Secondly, the isolated EPCs that contributes to postnatal neovascularization is heterogeneous and exhibits diversified morphological growth characteristics. As such, the genetic modification of EPCs presumably overcomes some of these limitations via altering the cell phenotype to improve the efficiency of cell-based therapeutic angiogenesis. Recently, efforts have been put to study the regulatory pathways that control the phenotypes of stem cell populations. Lots of these studies have concentrated on the role of the Wnt/b-catenin signaling pathway.

Wnts are a group of secreted, glycoproteins acting as short or long range signaling molecules, that bind to the receptors of the frizzled (Fz) family or to a co-receptor complex of Fz and several co-receptors such as lipoprotein receptor-related protein (LRP)-5/6, Ryk, or Ror2 [22]. The Wnt pathway is known to play important roles in multiple physiological and pathological processes [23]. Recent evidence suggests that the Wnt pathway is also important in the regulation of angiogenesis [24].

Therefore, we speculated that it might be reasonable to genetically manipulate the Wnt pathway in EPCs with adenovirus vectors to alter their vasculogenic properties in vitro and in vivo, and furthermore, discover some efficient therapeutic modulation applicable to clinical trials.

Materials and methods

Ethics

All animal experimental procedures were approved by institutional review board approved protocols and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

Isolation of EPCs

Mononuclear cells (MNC) were isolated from marrow of C57 mouse. Heparin was used as anticoagulant. MNC were isolated by density gradient centrifugation over Biocoll (Biochrom, Berlin, Germany) with PBS (Biochrom) buffer. Cells were either selected for the surface marker CD34 using anti CD34-coupled magnetic micro-beads (Fa. Miltenyi Biotech, Bergisch-Gladbach, Germany) as indicated by the company.

Culture of EPCs

Unselected MNC were planted on culture dishes coated with fibronectin (Sigma, Deisendorf, Germany) and cultured in endothelial cell growth medium (EGM, Clonetics, San Diego, USA) containing EGM SingleQuots: 12 μg/mL bovine brain extract (BEE), 10 ng/mL human epidermal growth factor (hEGF), 1 μg/mL hydrocortisone and antibiotics (Clonetics). This culture medium was supplemented with 5% fetal bovine serum (FBS, Sigma), 50 ng/ml human vascular endothelial growth factor (VEGF, Sigma),

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50 ng/ml human insulin-like growth factor-1 (IGF-1, Biomol, Hamburg, Germany) and an additional 50 ng/ml hEGF (Biomol). After 3 days non-adherent cells were removed and fresh culture medium was applied. Cultures were maintained through 9 days as others describe an endothelial phenotype occurring between 4th and 7th days. Phenotypical analysis of the cells was performed on 9th day.

**Flow cytometry analysis**

MNCs were harvested at passage four, and washed with 10% FBS/PBS and centrifuged at 1000 rpm, 5 min to gather a pellet. For flow cytometry analysis, MNCs were stained with PE-conjugated rat anti-CD31 and FITC-conjugated rat anti-CD133, at a concentration of 2 µg/mL at 4°C. Mouse IgG was served as negative controls. Unbound antibody was washed with 2 mL of 10% FBS/PBS after 30 min. Then pellets were re-suspended in 500 µL PBS and examined by flow cytometry with 10,000 events recorded for each condition. The results were analyzed by FACS Express software.

**Murine hind limb ischemic model and treatments**

Under sufficient anesthesia with sodium pentobarbital and local fur removal with depilatory cream, hind limb ischemia (HLI) was induced by entire ligation of the right femoral artery just below the inguinal ligament. In sham-operated, the suture was passed through but not tied. The murine model of HLI used for these experiments was based on that previously described by Couffinhal et al. [25]. Skin incision was performed at the middle portion of the left hindlimb overlying the femoral artery. The femoral artery then was gently isolated, and the proximal portion of the femoral artery was ligated with a 3-0 silk ligature. The distal portion of the saphenous artery was ligated, and other arterial branches as well as veins were all dissected free and excised. The overlying skin was closed using 2 surgical staples. After surgery, mice were kept on a heating plate at 37°C, and special care was taken to monitor the animals until they had completely recovered from anesthesia.

After surgery, the Murine HLI Model transgenic mice were equally divided into three groups (n=6): group 1 (transplanted with EPCs which injected with adenovirus vector), group 2 (transplanted EPCs plus injection with adenovirus vector encoding Wnt3a gene), group 3 (just model) and control with Sham operation. All treatments were initiated three days after the HLI procedure and continued for 28 days, local injection was performed on the same location and an immunosuppressor was administrated after cells transplanted to each group. On the 2nd, 14th and 21th, the blood flow ratio was measured using a laser Doppler perfusion imager (Moor Instruments, Devon, United Kingdom) to each group.

**qRT-PCR**

Total RNA was isolated from the cells by Trizol reagent (Invitrogen, USA). PCR was performed with a DNA thermal cycler in a 20 µl reaction volume, for 40 cycles via GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The mouse β-actin housekeeping gene was used as an internal control. The primers are as follows: VEGFa Forward Primer, 5'-CTGCCGTTCAAGTGGACCC-3', VEGFa Reverse Primer, 5'-CCCCT- CTTGTACCACTGTC-3', VEGFR Forward Primer, 5'-TTGGACAAATACACCCCTTCAGA-3', VEGFR Reverse Primer, 5'-GCTCCAGATATCTTCCAAAC-CA-3', β-catenin Forward Primer, 5'-CTGCCGG- GATGTTGGAAG-3', β-catenin Reverse Primer, 5'-CTCTCTCAGGAGCGAGATGCAA-3', β-actin Forward Primer, 5'-TGACGGTGACATCCGAAAAG-3', β-actin Reverse Primer, 5'-GCCGGAATCATCAGT ACTCC-3'.

**Western blotting**

The protein expression was measured by western blotting. The protein concentration of each sample was measured with a bicinechonic acid assay kit using BSA as standard (Pierce, Rockford, IL), anti-β-catenin (1:500, ab32572, abcam), anti-phospho β-catenin (1:500; ab-27798, Inc.), anti-Alpha Skeletal Muscle Actin (1:800; ab28052, abcam), anti-VEGFR2 (1:500, ab39256, abcam), anti-β-actin (1:1000, ab-8805, abcam). The secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin) was added at 1:2000 dilution. Peroxidase labeling was detected with the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech) and analyzed by a densitometry system. The relative protein level was normalized to β-actin (Santa Cruz Biotechnology, Inc.).
Elisa assay

Concentrations of VEGF and WNT3 in culture supernatants were determined by mouse ELISA kit (R&D Systems, USA) according to the manufacturer’s protocol.

Statistical analysis

Statistical analyses were performed with Statistic Package of Social Science (SPSS). Each point corresponds to mean ± SD. Statistical differences were determined by one-way or two-way analysis of variance (ANOVA) after post-hoc multi-comparison and P<0.05 was considered to be statistically significant.

Results

Isolation and characterization of EPCs

EPCs outgrown from mononuclear cells exhibited the exclusive “cobblestone” morphology growth pattern characteristic of endothelial cells. The endothelial phenotype of the outgrown EPCs was characterized by expression of endothelial markers (Figure 1), such as CD133 (90.8±5.5%), CD34 (81.6±4.8%) and negative for CD31 (9.9±1.3%).
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High-level expression of VEGFa and elevated expression of phosphorylated β-catenin in EPCs which were transfected with WNT3a gene in vitro

After selecting EPCs, the cells were transfected with adenovirus vector encoding Wnt3a gene, the high-level expression of Wnt3a was identified by western blot and RT-PCR (Figure 2A), it showed that the expression of Wnt3a in Wnt3a-transfected EPCs was three-fold more than in normal and control cells. To detect the expression of the stimulators belonging to angiogenesis such as VEGFa, Western blot with anti-VEGFa antibody and RT-PCR were also deployed (Figure 2B). Higher level of VEGFa was mark-
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edly detected in Wnt3a-transfected EPCs, the expression level of VEGFa was about four times higher than in normal and control cells. What’s more, western blot results indicated that vascular endothelial growth factor receptor-2 (VEGFR-2) and phosphorylated β-catenin were both elevated expressed in Wnt3a-transfected EPCs than normal and control EPCs (Figure 2C).

High-level expression of VEGFa and elevated expression of phosphorylated β-catenin in murine hind limb ischemic model by the treatment of WNT3a-transfected EPCs

After treatment of 28 days, we used RT-PCR and western blot for detecting β-catenin, VEGF, VEGFR mRNA in the three groups of Murine model.
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Figure 3 depicted that after transplanted with EPCs plus injected with adenovirus vector encoding Wnt3a gene, the muscular specimens of hind limb bear high-level expression of Wnt3a, elevated expression of VEGFR-2, as well as the phosphorylated β-catenin over expressed in treatment groups compared with control and normal group. Especially, Figure 3B showed that the β-catenin relative phosphorylation level was about four-fold than the model group and two times than control group, respectively. Similarly, the VEGFa expressed four-fold

Figure 4. Laser Doppler scanning of hind limb blood flow on the 21th day after CLI induction. (A) is simultaneous scanning of both hind limbs in sham-operated group, (B) is treatment of EPCs plus Wnt3a gene delivery group, (C) is treatment of EPCs only group, (D) is the CLI model group without EPCs and no Wnt3a gene delivery. (E) is chemia & normal perfusion ratio left to right in lower limb, #compared with Control. P<0.05.
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higher than in model group and about 1.5-fold higher than in control group. Furthermore, capillaries within skeletal muscle sections were visualized by immunostaining with antibody against VWF, 21 days after the induction of hind limb ischemia. Representative images demonstrated that EPCs plus Wnt3a gene delivery markedly increased capillary immunostaining (Figure 3D and 3E bottom panel).

Blood flow in the HLI region is improved with treatment

The blood flow of ischemic and normal did not show any difference among the six groups prior to the HLI procedure. But by day 21 after HLI induction, the blood flow was significantly reduced in the HLI group receiving no treatment (Figure 4D) compared with the other groups and was lower in the three groups receiving a single treatment of EPCs (Figure 4C) in comparison with the sham-operated, group (Figure 4A). Basically, the blood flow of EPCs plus Wnt3a gene delivery treatment groups recovered best among all the treatment groups and very close to the outcome of the sham-operated group (Figure 4E).

Discussion

Lower leg ischemia is the most severe form of peripheral arterial disease, which commonly affects the arteries supplying the leg and is mostly caused by atherosclerosis and is common in old age, putting patient at risk for heart attack and limb loss. The treatment options include injection of an anticoagulant, thrombolysis, embolectomy, surgical revascularization, or amputation. Especially, since 1997 Asahara T. et al., isolated endothelial cell (EC) progenitors from human peripheral blood and suggested its potential role of augmenting collateral vessel growth to ischemic tissues on the therapeutic angiogenesis, which is the natural healing process by which new blood vessels are formed on the basis of the cognate vascular bed to supply or increase oxygen-rich blood to the organ or part in deficit, many researchers have discovered that EPCs in peripheral blood, cord blood and bone marrow, stimulate the elevation of new blood vessels formation as well as promote the revascularization [26-28]. In comparison with traditional treatments, this technique can promote new blood vessels formation in HLI for some patients who are not eligible for surgery. Furthermore, for improving the treatment, some doctors have tried the technique which combined the EPCs with some proangiogenic factors such as VEGF, bFGF, Ang-2, PDGF-BB and HIF [29]. However, currently due to the insufficient knowledge on the detailed molecular mechanism and function of EPCs to the therapeutic angiogenesis, this technique has not been commonly used to clinical treatment throughout the world. In our preceding research, we found that after EPCs transfected with VEGF, the angiogenesis effects was improved, but the new-created capillaries was short of the periendothelial matrix and the pericyte. Thus, we believe that the periendothelial matrix and the pericyte/SMCs play the key role in therapeutic angiogenesis.

On the other hand, the Wnt pathway including three Wnt signaling pathways: the canonical Wnt pathay, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway, is known to play important roles in multiple physiological and pathological processes [23]. Emerging data shows that the Wnt pathway is very important in the regulation of angiogenesis [24]. Coincidently, in the downstream of canonical Wnt (also known as Wnt/β-catenin) pathway, the gene VEGF, which is also one important proangiogenic factor for EPCs, is scheduled to be activated [30]. Thus, a strong link has been established between the Wnt/β-catenin pathway and the activation as well as function of EPCs.

In this study, EPC were isolated from animal marrow cells and cord blood and transfacted with adenovirus vector encoding Wnt3a gene. Collectively, the genetically modified EPC displayed enhanced survival, proliferation, and differentiation in vitro. These cells also displayed augmented vasculogenic potential in vivo, suggesting that the Wnt pathway plays a regulatory role in the angiogenic function of EPCs.

To be specific, in our research the elevated expression of phosphorylated β-catenin was discovered both in Wnt3a-transfected EPCs (Figure 2C) and in murine HLI model by the treatment of EPCs plus Wnt3a gene delivery (Figure 3A). Our research also discovered that VEGFa, highly expressed in Wnt3a-transfected EPCs (Figure 2B) and in murine HLI model by
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the treatment of EPCs plus Wnt3a gene delivery (Figure 3C). Among angiogenic factors reported so far, VEGFα is the mediator for the basic signaling of angiogenesis, particularly signals for endothelial cell growth in vivo [31]. VEGFα belongs to the platelet derived growth factor (PDGF) supergene family, and as a homodimer, binds to, and activates two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 [32], our in vitro and in vivo experiments also exhibited that the expression of VEGFR-2, the major receptor of VEGFα, significantly elevated in EPCs (Figure 2C) and in murine HLI model by the treatment of EPCs plus Wnt3a gene delivery (Figure 3A).

These results indicated that during the angiogenesis derived from EPCs, the over-expression of Wnt3a stimulated the Wnt/β-catenin pathway, led to increased nuclear translocation of β-catenin and increased secretion of angiogenic cytokines (VEGFα). It enhanced the survival, proliferation as well as promoted the differentiation of EPCs, finally initiated the high growth of periendothelial matrix and the pericyte/SMCs as to increasing the potency of EPCs for therapeutic vasculogenesis. Transplantation of these EPCs plus injection of adenovirus vector encoding Wnt3a gene into the HLI model of mouse significantly improved blood flow and tissue capillary density compared with model group (only EPCs treatment group).

However, a potential problem with the genetic therapy for vasculogenesis is insufficiency of EPC numbers for clinical applications. In the coming research, we will focus on finer cell regulation network on the therapeutic angiogenesis in hope to significantly improve the efficiency of EPCs to overcome the limitation of EPCs number.

In conclusion, our data extend the knowledge on the regulatory role of Wnt/β-catenin signaling pathway in therapeutic angiogenesis, lay a foundation for exploring the detailed molecular mechanism on the formation of capillarity network of blood vessels during therapeutic angiogenesis, which may expand the potential applications of this therapeutic modulation for CLI patients.

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Disclosure of conflict of interest

None.

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