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Interleukin-22 receptor 1 upregulation and activation in hypoxic endothelial cells improves perfusion recovery in experimental peripheral arterial disease

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ABSTRACT

Objective: Inflammation induced by muscle ischemia is involved in tissue repair and perfusion recovery in peripheral arterial disease (PAD) patients. Interleukin (IL)-22 is an inflammatory cytokine discovered in recent years and shows versatile functions; however, its role in PAD remains unknown. Here, we test whether IL-22 and its receptors are involved in angiogenesis in experimental PAD.

Methods and results: Both IL-22 and its receptor—IL-22 receptor 1(IL-22R1) were upregulated in muscle and endothelial cells after ischemia. In experimental PAD models, blocking IL-22 using IL-22 monoclonal antibody impaired perfusion recovery and angiogenesis; on the other hand, IL-22 treatment improved perfusion recovery. Ischemic muscle tissue was harvested 3 days after experimental PAD for biochemical test, IL-22 antagonism resulted in decreased Signal Transducer and Activator of Transcription (STAT3) phosphorylation, but did not alter the levels of VEGF-A or cyclic guanine monophosphate (cGMP) levels in ischemic muscle. In cultured endothelial cells, IL-22R1 was upregulated under simulated ischemic conditions, and IL-22 treatment increased STAT3 phosphorylation, endothelial cell survival and tube formation. Knock down of IL-22R1 or treatment with STAT3 inhibitor blunted IL-22-induced endothelial cell survival or tube formation.

Conculsion: Ischemia-induced IL-22 and IL-22R1 upregulation improves angiogenesis in PAD by inducing STAT3 phosphorylation in endothelial cells. IL-22R1 may serve as a new therapeutic target for PAD.

1. Introduction

Peripheral arterial disease (PAD) is caused by occlusions of the arteries to lower extremities, affects more than 150 million people worldwide and puts them at risk for lower extremity amputation and even death [1–5]. Since total occlusions of the major inflow artery to the leg(s) is common in PAD, the quantity of blood that can be delivered to the distal tissue becomes dependent on the extent of angiogenesis which is important to rebuild vascular network and recover blood perfusion to the ischemic leg [6–8].

Recent studies indicate that inflammation plays an important role in angiogenesis and vascular remodeling after PAD [9,10]. Interleukins (IL) are a group of cytokines that participate in communication among leukocytes and other cells, and regulate numerous biological inflammatory processes. A number of studies suggest that several interleukins (IL) are involved in angiogenesis and tissue recovery after limb ischemia in PAD [10–15]. IL-22 belongs to the IL-10 cytokine family and plays both pro-inflammatory and anti-inflammatory roles via modulating secretion of inflammatory mediators [16], and IL-22 transduce its signal through the cell membrane receptors which include IL-22R1 and IL-10R2 [16]. Because of its versatile functions, IL-22 has generated considerable interest in recent years [16,17]. Recent reports shows that IL-22

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promotes angiogenesis in tumors. However, its role in PAD has not been known. This study is aimed to investigate the effects of IL-22 on the process of angiogenesis and perfusion recovery after PAD.

2. Materials and methods

2.1. Hindlimb ischemia model and IL-22 modulations

Male Balb/c mice (25–30 g) and C57BL/6 mice (25–30 g) (HFK Bioscience, Beijing, China) were housed in a pathogen-free mouse room in Renmin Hospital of Wuhan University during the study. Mouse hindlimb ischemia were used as a preclinical PAD model, surgery of hindlimb ischemia were described as previously [18]. Briefly, unilateral femoral artery ligation and excision were performed on the left side of mice after anesthesia induction (phenobarbital 30–40 mg/kg), Expression levels of IL-22R1 and IL-10R2 were detected in the hindlimb 7 days after HLI. Immediately after surgery, Balb/C mice were treated with vehicle (50 μL, n = 12), recombinant mouse IL-22 (rIL-22; 15 μg/kg, n = 12), C57BL/6 mouse were treated with *anti*-IL-22 neutralizing monoclonal antibody (*anti*-IL-22 mAb; 1.25μg/mice, n = 12) or an equivalent amount of isotype IgG (n = 12). All the treatments were intraperitoneally injected. All procedures involving animal use conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health, and the protocol was approved by the Institutional Animal Care Committee from Wuhan University, PR China.

2.2. Perfusion recovery

Perfusion flow in the ischemic and contralateral non-ischemic limbs was measured as with the use of a laser Doppler perfusion imaging system (Perimed, Inc, Ardmore, PA, USA) [19] [19 21 23 19 19 19 19, 20]. Perfusion was expressed as the ratio of left (ischemic) to right (non-ischemic) hind limb and was performed on days 0, 7, 14, 21 and 28 after surgery. In mice that developed autoamputation, the perfusion ratio obtained from the limb before autoamputation was used.

2.3. Immunofluorescence

For assessment of capillary density, 28 days post-HLI, ischemic gastrocnemius muscle sections from mice were used for immunofluorescent staining as described previously [18]. Briefly, anti-CD31 antibody (rat anti-mouse CD31 Ab; cat: # 550274; BD Pharmingen San Jose, CA) was applied on acetone-fixed cryosections of ischemic gastrocnemius muscle specimens at 4 °C overnight in blocking solution. After rinsing with Phosphate Buffered Saline (PBS), Alexa Fluor 488 phalloidin (used for muscle fiber staining, ThermoFisher scientific, Waltham, MA) and secondary reagents Goat anti-rat Alexa Fluor 555 (1:100, Invitrogen), were applied for 1 h at room temperature. Secondary antibody only, without primary antibody, was used as a negative control to assess non-specific binding. Stained sections were examined with 200× magnification, using an Olympus I×71 high-magnification microscope. Assessment of capillary densities were analyzed by counting 5 random high-power (magnification×200) fields, and was expressed as the number of $CD31^+$ cells per muscle fiber.

2.4. RNA isolation and quantitative PCR

Total RNA was isolated and used for real-time quantitative RT-PCR (qPCR) as previously described [20,21]. qPCR was performed using primer/probes for *il22r1*, *il10r2* and 18S from Applied Biosystems (Foster City, CA). Quantitative normalization of cDNA in each sample was performed using expression of 18S rRNA as an internal control. The generated Ct value of each gene was normalized by its respective Ct value of 18S rRNA (ΔCt). Each gene was then further normalized to the average ΔCt value of its control group (ΔΔCt). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

2.5. Western blotting

Mouse muscle tissue and cells were homogenized in RIPA lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, Shanghai, China) as described previously [22]. Equal amounts of protein in homogenate samples were separated in SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA) and blotted with primary antibodies and corresponding peroxidase-conjugated secondary antibodies. Primary antibodies against phosphorylated signal transducer and activator of transcription 3 (p-STAT3, Y705), STAT3, beta actin (sc-47778), and secondary antibodies were obtained from Cell Signaling (Danvers, MA, USA) or Santa Cruz (Santa Cruz, CA, USA) Company. The bound antibody signal was developed using an Immun-Star HRP chemiluminescent kit (Shanghai Tanon Science & Technology, Shanghai, China). Western blot image was obtained by Tanon 5200 Chemiluminescence Imaging System (Shanghai Tanon Science & Technology). Semi-quantitative analyses of immunoblots was performed using the Image J.

2.6. Cell culture and in-vitro angiogenesis assay

Human umbilical vein endothelial cells (HUVEC) were isolated from a donor umbilical vein as described previously [23], and then grew in endothelial cell growth medium (Cell Applications Inc, San Diego, CA) supplemented with 10% fetal bovine serum (FBS, Gibco, Shanghai, China). To mimic the endothelial cells under ischemic conditions in HLI models, HUVECs were exposed to hypoxia (2% oxygen, BioSpherix, Lacona, NY) and serum starvation (HSS). The use of human umbilical vein was approved by Wuhan University Institutional Review Boards.

In vitro transfection of siRNA for IL22R1 in HUVECs were as described previously [22]. Briefly, a reverse transfection protocol using neofx transfection agent (Ambion, Austin, TX) was used to transfect IL22R1 siRNA, or scrambled RNA (Life Tech, San Jose, CA, USA) into HUVECs for 48 h.

For cellular viability studies, HUVECs transfected with siRNA or scrambled RNA were plated in a 96-well plate at a density of 1×10^4 cells/well (n = 8/group), and then cultured under HSS conditions with or without IL-22 (10 ng/mL) for 48 h. At the end of the incubation, cell viability was assessed using tetrazolium dye incorporation (BioVision, Milpitas, CA).

In-vitro angiogenesis assay were performed as previously described under HSS conditions [19]. Briefly, HUVECs transfected with IL22R1 siRNA, or scrambled RNA were plated at a density of 1×10^4 cells/well on 96-well dishes which were coated with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA), and then exposed to HSS conditions with or without IL-22 for 6 h to assess tube formation. Each condition was performed in 8 wells. The degree of tube formation was determined by measuring the number of loops from each well under 40× magnifications using Image J (National Institute of Health, Bethesda, MD). Each experiment was repeated at least in two different batches of HUVECs.

2.7. Enzyme-linked immunoassay (ELISA)

For the detection of IL-22, vascular endothelial growth factor-A (VEGF-A) and cyclic guanosine monophosphate (cGMP). Protein

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