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Total saponins of panaxnotoginseng promotes lymphangiogenesis by activation VEGF-C expression of lymphatic endothelial cells

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Chloroform (PubChemCID:6212)
Methanol (PubChemCID:887)
Acrylamide (PubChemCID: 6579)
Ammonium Persulfate (PubChemCID:62648)
Glycine (PubChemCID:750)
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ABSTRACT

Ethnopharmacological relevance: Lymphatic system plays an important role in maintaining the fluid homeostasis and normal immune responses, anatomic or functional obstruction of which leads to lymphedema, and treatments for therapeutic lymphangiogenesis are efficiency for secondary lymphedema. Total saponins of panaxnotoginseng (PNS) are a mixture isolated from Panaxnotoginseng (Burkill) F.H.Chen, which has been used as traditional Chinese medicine in China for treatment of cardio- and cerebro-vascular diseases. The aim of this study was to determine the effect and mechanism of PNS on lymphangiogenesis.

Methods: The Tg (fli1: egfp; gata1: dsred) transgenic zebrafish embryos were treated with different concentrations of PNS (10, 50, 100 μM) for 48 h with or without the 6 h pretreatment of the 30 μM Vascular endothelial growth factors receptor (VEGFR)-3 kinase inhibitor, followed with morphological observation and lympangiogenesis of thoracic duct assessment. The effect of PNS on cell viability, migration, tube formation and Vascular endothelial growth factors (VEGF)-C mRNA and protein expression of lymphatic endothelial cells (LECs) were determined. The role of phosphatidylinositol-3 (PI-3)-kinase (PI3K), extracellular signal-regulated kinase (ERK)1/2 pathways, c-Jun N-terminal kinase (JNK) and P38 mitogen activated protein kinases (MAPK) signaling in PNS-induced VEGF-C expression of LECs by using pharmacological agents to block each signal.

Results: PNS promotes lymphangiogenesis of thoracic duct in zebrafish with or without VEGFR3 Kinase inhibitor pre-impairment. PNS promotes proliferation, migration and tube formation of LECs. The tube formation induced by PNS could be blocked by VEGFR3 Kinase inhibitor. PNS induce VEGF-C expression of LEC, which could be blocked by ERK1/2, PI3K and P38MAPK signaling inhibitors.

Conclusion: PNS activates lymphangiogenesis both in vivo and in vitro by up-regulating VEGF-C expression and activation of ERK1/2, PI3K and P38MAPK signaling. These findings provide a novel insight into the role of PNS in lymphangiogenesis and suggest that it might be an attractive and suitable therapeutic agent for treating secondary lymphedema or other lymphatic system impairment related disease.

1. Introduction

Lymphatic system plays an important role in maintaining the fluid homeostasis and normal immune responses, which transport extravasated fluid and macromolecules from peripheral tissues, filter lymphatic fluid and remove foreign material. Anatomic or functional obstruction of the lymphatic system leads to the progressive accumulation of protein-rich fluid in the interstitial spaces, which is named as lymphedema (de Almeida and Freedman, 1999; Szuba and Rockson, 1998). The condition can be inherited or resulting from trauma, surgery, radiotherapy, or parasitic infection (secondary lymphedema). In industrialized countries, cancer treatment is the leading cause of secondary lymphedema (DiSipio et al., 2013). Furthermore, 10–30% of patients with malignant tumor develop lymphedema (Beesley et al., 2007; Cormier et al., 2010; Ohba et al., 2011; Tada et al., 2009). Despite significant progress in surgical and conservative techniques, therapeutic options for the treatment of lymphedema are limited (Ko et al., 1998; Saito et al., 2013; Szuba and Rockson, 1998). Although

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rarely lethal, lymphedema is a disfiguring and disabling condition, which reduces the quality of life (Girgis et al., 2011). There is no cure for lymphedema currently (Ostby and Armer, 2015). Several preclinical experiments demonstrated that treatments for therapeutic lymphangiogenesis are efficiency for secondary lymphedema (Cheung et al., 2006; Yoshida et al., 2015; Zhou et al., 2011). Enhancement of lymphangiogenesis in situations of lymph accumulation is considered as a promising strategy.

Lymphangiogenesis can be stimulated by various cytokines, but vascular endothelial growth factors (VEGF)-C is the most important and specific lymphatic vessel growth factors known. VEGF-C binds VEGF receptor-3 (VEGFR-3), which is expressed on lymphatic endothelial cells (LECs), and promotes mainly lymphangiogenesis (Khadim et al., 2015). It was reported that VEGF-C-deficient mice are unable to develop a functional lymphatic system (Karkkainen et al., 2004), transgenic expression of soluble VEGFR-3 results in inhibition of lymphangiogenesis and pronounced lymphedema (Makinen et al., 2001), and gene transfer of VEGF-C effectively augments lymphangiogenesis and ameliorates lymphedema in animal models (Yoon et al., 2003; Zhou et al., 2011). Therefore, VEGF-C is a valuable therapy target for lymphangiogenesis and lymphedema.

Panaxnotoginseng (Burkill) F.H.Chen, named as Sanqi in China, has been used as traditional Chinese medicine in China, for treatment of cardio- and cerebro-vascular diseases, such as Intracranial/intracerebral hemorrhage of stroke, ischemic heart and brain diseases, inflammation, trauma, and internal and external bleeding due to injury for thousands of years (Chen, 1987). Total saponins of panaxnotoginseng (PNS), a mixture isolated from Panaxnotoginseng, whose major components include ginsenosides and notoginsenosides (Yao et al., 2011), were the biologically active constituents responsible for the therapeutic action of this medicine (Park et al., 2009; Zhang et al., 2007). Previously, we screened several extracts from herbs for their ability to promote lymphangiogenesis in zebrafish, and found that PNS has such ability. Thus, the aim of our current study is to investigate that whether and why PNS could promote lymphangiogenesis.

2. Materials and methods

2.1. Chemicals

Noto-G™ extracts from the root of Panaxnotoginseng (Burkill) F.H.Chen were supplied by National Institutes for Food and Drug Control (NIFDC) (Lot number: 110,870–201002). Notoginseng was extracted from the root of the plant using ethanol and standardized to contain notoginsenoside R1 6.9%, ginsenosides Rg1 28%, ginsenoside Re 3.8%, ginsenoside Rb1 29.7%, ginsenoside Rd 7.3% of the whole extract, respectively. The quantification of total saponins of panaxnotoginseng in the notoginseng extract was determined by high-performance liquid chromatography analysis by NIFDC (Supplementary Fig. 1). The extract was dissolved in embryo water for zebrafish or culture grade DMSO (Sigma–Aldrich, St. Louis, MO) for murine LEC and subsequently sterile-filtered through a 0.22 μM Millipore membrane. PD98059 (cat. #S1177), Wortmannin (cat. #S2758), SB203580 (cat. # S1076) and SP600125 (cat. # S1460) were purchased from selleckchem.

2.2. Animals

The transgenic zebrafish line (fli1: egfp; gata1: dsred), which expresses eGFP at endothelial cells and dsred at blood cells (Omae et al., 2013; Serbanovic-Canic et al., 2011), was kindly provided by Simon Ming Yuen Lee (Institute of Chinese Medical Sciences, Macau SAR.). It was maintained in zebrafish room of Longhua hospital, a controlled environment according to the description in the Zebrafish Handbook (Westerfield, 1995). Embryos were generated by natural pair wise mating, and were raised at 28.5 °C in embryo water (13.7 mM NaCl, 540 μM KCl, pH 7.4, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 300 μM $CaCl_2$, 100 μM $MgSO_4$, 420 μM $NaHCO_3$, pH 7.4). All animal experiments were conducted according to the ethical guidelines of Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine.

2.3. Drug administration

At 48 h post fecundation (hpf), healthy zebrafish embryos were picked out and were distributed into a 12-well microplate with 10 fishes per well. Following this, the embryos were treated with different concentrations (10, 50, 100 μM) of PNS (National Institutes for Food and Drug Control, CAS No: 88,105-29-7, Lot No: 110,870–201002, purity > 98.5, 80 mg/kg) or 0.2%DMSO as a vehicle control for 48 h with or without the pretreatment of the 30 μM VEGFR-3 kinase inhibitor (MAZ51, Calbiochem, La Jolla, CA, cat. #676492, lot. #D00152431) for 6 h. Each group had more than 9–10 fishes.

2.4. Morphological observation and quantification of lymphatic phenotype of zebrafish

Zebrafish embryos were anesthetized, plated and oriented laterally on a coverslip after treatment. Image acquisition from zebrafish embryos was achieved using a Confocal Fluorescence Imaging Microscope (Leica TCS-SP5, Germany) and merged Z-stack images by visualization 3D projection. The length of developing lymphatic thoracic ducts (TD) of zebrafish embryos was individually counted from the trunk region spaning 10 somites, from somite boundary 7 or 8–18, on merged Z-stacked confocal images. Experiments were performed in triplicate. Quantification graphs were generated by Leica Application Suite Advanced Fluorescence 2.3.6 build 5381 program.

2.5. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

A murine LEC cell line established from Freund's adjuvant-induced benign lymphangiomas (Sironi et al., 2006) (Cells were provided by Dr. S. Ran from the University of Illinois, USA). Proliferation of LECs was examined by MTT assay (MTT based Cell Growth Determination Kit, Sigma) according to the manufacturer's instructions. In brief, cells were seeded at a density of 1×10^4 cells/well in 96-well plates in quadruple. Twenty four hours later, cells were treated with different concentrations of PNS (0, 10, 50, 100 μM) or VEGF-C (Sigma, cat. #SRP6020, lot. #MMS1713061, 0.34 nM) for another 72 h. Then cells were incubated with 20 μl of MTT solution at 37 °C for 4 h, followed by 200 μl of MTT solvent to terminate the reaction. The plates were read at 570 nm using a benchmark microplate reader (BioRad).

2.6. Wound healing assay

Murine LECs (2×10^5 cells/well) were cultured in 12-well plate. A confluent monolayer of Cells was wounded with a yellow pipet tip and the media was replaced by culture medium containing different concentration of PNS (0, 10, 50, 100 μM) or VEGF-C (0.34 nM). Closure of the wound was monitored and representative photomicrographs were taken at 24 h after PNS or VEGF-C treatment. A reduction in the scraped area indicates a sign of migration. The migration length= scraped distance at 0 h - scraped distance at 24 h.