Qingre Huoxue Decoction, A Traditional Chinese Herbal Formulation, Impacts Angiogenesis in Psoriasis-Like Skin Through the HIF-1 α /Flt-1/VEGF Pathway

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Abstract

Background: Qingre Huoxue Decoction (QHD), a traditional Chinese medicine (TCM) formulation, could alleviate psoriasis in our previous studies. The present work aimed to assess QHD's effects on psoriasis and the underpinning mechanism in cultured cells and experimental animals.

Methods: The CCK-8 assay was carried out for cell viability assessment. HUVEC migration was assessed by transwell and wound healing assays. QHD-induced suppression of capillary tube formation in HUVECs was detected by tube formation assay. In addition, the imiquimod (IMQ)-induced male BALB/c mouse model of psoriasis was established to examine the Psoriasis Area and Severity Index (PASI) after QHD administration. HIF-1 α , Flt-1 and VEGF expression levels *in vivo* were assessed by immunoblot, qPCR and immunofluorescence.

Results: The results showed that QHD dose-dependently reduced viability in HUVECs. In addition, QHD suppressed tube formation in HUVECs at levels below those needed to inhibit HUVECs. Upon QHD administration, HUVEC migration was markedly decreased; QHD effectively prevented the migratory ability of HUVECs, as determined by wound areas at 0h, 12h and 24h, respectively. Finally, QHD starkly downregulated HIF-1 α , Flt-1 and VEGF in the IMQ-induced mouse model, at the protein and mRNA levels.

Conclusions: In summary, QHD inhibits angiogenesis in cultured cells and mice. HIF-1 α /Flt-1/VEGF signaling is important in angiogenesis and psoriasis development. These findings provide a rationale for developing QHD for clinical use against psoriasis.

Introduction

Psoriasis represents an incurable chronic inflammatory skin disease affecting about 3% of all humans¹. In Caucasians, an incidence of about 100,000 people per year has been reported. Histologically, psoriasis mostly features enhanced angiogenesis, unrestrained growth, abnormal keratinocyte differentiation and high amounts of infiltrated immune cells^{2,3}. However, how psoriasis is initiated remains unknown. It was suggested that angiogenesis represents a hallmark of psoriasis and psoriatic arthritis⁴. Meanwhile, angiogenesis is accompanied by enhanced endothelial cell (EC) proliferation, adhesion and migration⁵.

Critical angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptor Flt-1, show increased amounts in human psoriasis skin⁶. Mounting evidence indicates vascular endothelial growth factor (VEGF) upregulation constitutes an early and essential factor in psoriasis pathogenesis. It was recently demonstrated hypoxia-induced VEGF is a hallmark of psoriasis. In agreement, after psoriasis treatment with a TNF inhibitor, VEGF and Flt-1 are downregulated in P-DMSCs, and angiogenesis is reduced in the skin lesion. Hypoxia is characteristic of the skin tissue in patients with psoriasis, with HIF in keratinocytes and endothelial cells promoting angiogenesis by increasing the expression of VEGF and Flt-1, respectively. In psoriasis, abnormal rapid cell proliferation increases oxygen

consumption, while epidermal thickening reduces oxygen supply. It was suggested local hypoxia in skin might be critical for adaptive upregulation of angiogenic proteins, including hypoxia inducible factor-1 alpha (HIF-1 α).

The traditional Chinese herbal formulation Qingre Huoxue Decoction (QHD) could alleviate psoriasis in our previous work. QHD is mainly composed of *Sophora japonica* (Huaihua), *Angelica sinensis* (Danggui), *Sparganium stoloniferum* (Sanleng), *Curcuma zedoaria* (Ezhu) and *Spatholobus suberectus* Dunn (Jixu eteng). The main pharmacologically relevant constituents include Rytub (Huaihua), ferulic acid (Danggui), isocurcumenol (Ezhu), hexadecanoic acid (Sanleng) and formononetin (Jixueteng). To determine whether QHD modulates angiogenesis by regulating hypoxia during psoriasis treatment, the imiquimod-induced psoriasis-like mouse model was used. Angiogenesis and related genes were detected in mouse psoriasis-like lesions induced by imiquimod. The present work aimed to assess QHD's effects on psoriasis and the underpinning mechanism in cultured cells and experimental animals.

Methods

All assays involving animals had approval from the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Drug preparation

Drug components were provided by Jiangyin Tianjiang Pharmaceutical (China). After boiling the herbs, the filtrates underwent concentration and drying under vacuum at 60°C. The resulting extract was further lyophilized to produce QHD (yield of 27.5%)⁷. High performance liquid chromatography (HPLC) was utilized for measuring the main constituents. The drug composition ratios of *Sophora japonica* (Huaihua), *Angelica sinensis* (Danggui), *Sparganium stoloniferum* (Sanleng), *Curcuma zedoaria* (Ezhu) and *Spatholobus suberectus* Dunn (Jixu eteng) in QHD were 40, 20, 20, 10 and 10%, respectively. QHD was solubilized with PBS. The final extract (stored at 4°C), was submitted to quality control per the guidelines published by Chinese State Food and Drug Administration (SFDA).

Cells and culture

Human Umbilical Vein Endothelial Cell (HUVEC; Lonza, Belgium) culture was carried out with endothelial basal medium (EBM) containing the components of Endothelial cell growth medium-2 Bullet Kit (Lonza) according to instructions. All cells were cultured at 37°C in a humid environment containing 5% CO₂.

Animal experiments

Assays involving animals had approval from the ethics committee of Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine. Totally 25 SPF BALB/c male mice, 18 $\pm$ 2g, were provided by Shanghai Slac Laboratory Animal (China; license SCXK [Shanghai] 2008-0016) and housed in Shanghai University of Traditional Chinese Medicine (laboratory license, SCHK [Shanghai] 2021-0016).

The twenty-five animals were randomized into five groups, including the IMQ (IMQ, psoriasis model), IMQ+QHD low-dose (IMQ+L, 8 mg/kg/day), IMQ+QHD medium-dose (IMQ+M, 16 mg/kg/day), IMQ+QHD high-dose (IMQ+H, 32 mg/kg/day) and methotrexate (MTX, 1 mg/kg/day) groups. Totally 42 mg of IMQ cream was administered daily to the animals (back skin) for 7 days. Erythema and scaling were scored at 0, 2, 4 and 7 days, respectively, as 0 (none), 1 or 2 (moderate), 3 (severe) and 4 (very severe) according to the Psoriasis Area Severity Index (PASI). On the eighth day, euthanasia was performed by intravenous injection of excess pentobarbital 100 mg/kg, followed by skin sample collection for hematoxylin and eosin (H&E) staining and Western blot. Figure.3 shows the experimental design.

The CCK-8 assay

The CCK-8 assay was carried out for cell viability assessment. Cell seeding was performed in 96-well plates at 10^5 cells per well followed by a 24-h incubation at 37°C prior to the administration of QHD at 0, 25, 50, 100, 150 and 200 µg/ml for 48h. Post-treatment, 0.5 mg/ml CCK-8 solution (100 µl per well) was supplemented for a 2-h incubation. OD was read on a Varioskan Flash multiscan plate reader (Thermo, USA) at 450 nm. Triplicate assays were carried out.

Transwell migration assay

HUVECs migration was assessed in 24-well Transwell plates using 8-µm-pore polycarbonate membrane inserts (Corning, USA) with Matrigel (BD Biosciences) coating as directed by the manufacturer. Briefly, 3×10^5 cells in 100 µl medium were placed in each upper compartment for a 24-h incubation at 37°C. Cells detected on the lower surface of the membrane by hematoxylin staining were numbered in five randomly selected high-power fields. Triplicate assays were carried out.

Scratch assay

The scratch assay was performed to further examine HUVEC migration as reported previously⁸. In brief, cells were treated for 48h and a sterile 200-µl micropipette tip was used for scratching the well's center. Upon washing with PBS, EBM with 1% FBS (Gibco Life Technologies, USA) was supplemented. The samples were incubated at 37°C, and gaps were examined at 0, 12 and 24h, respectively, using an inverted microscope equipped with an Olympus DP70 camera (Olympus, Japan). Image J was utilized for quantitation.

Tube formation assay

HUVECs were examined for tube formation with Geltrex-reduced growth factor basement membrane matrix (Invitrogen), as directed by the manufacturer. For quantifying tube formation, an inverted microscope (Olympus) was utilized to count meshes which were further analyzed with Image J.

Real-time quantitative PCR

Total RNA extraction utilized TRIzol reagent (Invitrogen) as directed by the manufacturer. A reverse transcription kit from Takara was used for cDNA synthesis, followed by quantitative PCR with SYBR green (Takara). The following primers were utilized: HIF-1 α , sense 5'-CATCTCCATCTCCTACCCACAT-3' and antisense 5'-ACTCCTTTTCCTGCTCTGTTTG-3'; VEGF, sense 5'-TGGGCTCTTCTCGCTCCGTAGTAG-3' and antisense 5'-GCCGCCTCACCCGTCCAT-3'; Flt-1, sense 5'-AAAGGCTGAGCATCACTCCC-3' and antisense 5'-CTACAGGTGTAGAGGCCCGT-3'. The $2^{-\Delta\Delta Ct}$ method was used for the analysis of triplicate assays.

Immunoblot

Prior to protein extraction, imiquimod (IMQ)-induced mice were treated with QHD for 24h. Total protein was extracted from IMQ-induced mouse samples using a protein extraction kit (Applygen Technologies, China) as directed by the manufacturer. Protein separation was carried out by 12% SDS-PAGE, followed by transfer onto NC membranes. These membranes underwent overnight incubation (4°C) with anti-HIF-1 α (1:1000, Novus Biologicals, USA), anti- β -actin (1:5000, Cell Signaling Technology, CST, USA), VEGF (1:5000, CST) and Flt-1 (1:2000, CST), respectively. This was followed by treatment with a 24-h incubation with secondary antibodies at 4°C. The enhanced chemiluminescence system was utilized for detection. Densitometric analysis was performed with the Quantity One image analysis software (Bio-Rad, USA).

Statistical analysis

GraphPad Prism 6 (GraphPad Software, USA) was used for data analysis. Student's *t* test and Wilcoxon's signed rank test were performed for comparisons. *P*<0.05 reflected statistical significance. Data are mean $\pm$ standard deviation (SD).

Results

QHD inhibits proliferation and capillary tube formation in HUVECs

We evaluated QHD's effects on HUVEC proliferation by the CCK-8 assay. It was found QHD dose-dependently reduced proliferation in HUVECs (Figure.1A). Following a 24-h treatment, QHD's IC₅₀ in HUVECs averaged 471.5 μ g/ml. Primary HUVECs incubated for 24 adhered to the plate. Following a 3 to 5-day incubation, corroborating previously reported findings, HUVECs showed cobblestone-like morphology. What's more, the angiogenic capability of HUVECs was dose-dependently altered by QHD. QHD at 20, 200 and 400 μ g/ml for 24h, respectively, markedly reduced tube formation in HUVECs in comparison with untreated cells (*P*<0.05) (Figure.1B). Jointly, the above data demonstrated QHD suppressed tube formation in HUVECs at levels starkly lower than needed for inhibiting HUVECs. Remarkably, QHD promoted capillary tube formation in HUVECs.

QHD suppresses HUVEC migration *in vitro*

Endothelial cell migration is important in neovascularization, and involves chemotactic, haptotactic and mechanical components. HUVEC migration was first assessed by the transwell assay (Figure.2A). In comparison with control cells, the QHD groups of HUVECs had starkly reduced migration. Next, HUVECs were examined for mechanical migration by the scratch assay. At 0, 12 and 24h upon wounding, respectively, shorter distances were travelled by HUVECs after QHD treatment (Figure.2B). These data also demonstrated that QHD effectively reduced the migratory ability of HUVECs.

QHD impacts angiogenesis through the HIF-1 α /Flt-1/VEGF pathway in the imiquimod (IMQ)-induced mouse model

In the process of angiogenesis, HIF-1 α /Flt-1/VEGF signaling plays a critical role. To further assess QHD's effects, the IMQ-induced mouse model was established. At 3-5 days, mouse recombinant QHD was given by gavage. On the seventh day, psoriasis-like skin inflammation was observed as reflected by erythema and scaling (Figure.3). In this study, QHD-reduced angiogenesis was investigated using dorsal skin images of BALB/c mouse psoriasis models (Figure.3).

Skin samples from animals administered QHD at 200 and 400 μ g/kg were thinner, with reduced neovascularization compared with the model group. Its effect was similar to that of the positive control drug. To detect the cell expression levels and localizations of HIF-1 α and Flt-1 in the IMQ-induced mouse model, immunofluorescence was performed (Figure.4A-B). In addition, HIF-1 α , Flt-1 and VEGF protein levels in the IMQ-induced mouse model were assessed by Western blot (Figure.4C). Finally, HIF-1 α , Flt-1 and VEGF mRNA amounts in the IMQ-induced mouse model were assessed by qRT-PCR (Figure.4D-F). As shown in Figure.4, QHD remarkably inhibited the protein and mRNA expression levels of these effectors in the IMQ-induced mouse model.

Discussion

It was found that local hypoxia is an important feature of psoriasis. Hypoxia is a major factor in the early pathogenetic events of psoriasis, contributing to inflammatory cell recruitment; meanwhile, the cross-talk involving epidermal and immune cells likely control psoriasis development. Therefore, adjustment of the hypoxic microenvironment is an important direction in the treatment of psoriasis. Angiogenesis is one of the major mechanisms of tissue adaptation to hypoxia.

It is admitted local hypoxia in skin is essential in the adaptive induction of angiogenesis, upregulating multiple effectors such as hypoxia inducible factor-1 alpha (HIF-1 α). Multiple reports have revealed HIF-1 α is upregulated in psoriatic skin lesions and in blood.

HIF-1 α is a heterodimeric transcriptional complex comprising the HIF-1 α and HIF-1 β subunits, with a function in controlling oxygen levels^{9, 10}. However, HIF-1 was recently shown to affect other stress response events beside hypoxia. Meanwhile, HIF-1 α amounts are elevated in psoriatic lesions^{11, 12}, and in serum specimens from psoriasis cases. These elevated HIF-1 α amounts alongside a high association of HIF-1 α upregulation with disease severity suggest a major role for HIF-1 α in psoriasis' pathogenetic

mechanisms¹³. Therefore, angiogenesis, exaggerated proliferation and inflammatory response might constitute a vicious circle, with HIF-1 α helping maintain and amplify this vicious circle. In agreement, drugs controlling HIF-1 α expression might be efficient in disease management. HIF-1 α could represent an important target to control inflammatory response and angiogenesis, since many effectors in various pathways seem to involve HIF-1 α .

Traditional Chinese medicinal (TCM) preparations have been effectively utilized in treating psoriasis for years. Recently, angiogenesis in psoriasis was shown to be suppressed by TCM preparations. The present study showed QHD inhibits proliferation, migration and tube formation in cultured HUVECs. The present data also demonstrated QHD can significantly reduce the PASI in IMQ-induced mouse models. Multiple preliminary works showed angiogenesis is associated with the HIF-1 α /Flt-1/VEGF pathway. The IMQ-induced mouse model was established to further explore the mechanism underpinning the inhibitory effects of QHD on psoriasis. The present *in vivo* study showed that QHD effectively suppresses angiogenesis, which is associated with the HIF-1 α /Flt-1/VEGF pathway.

Conclusions

In summary, this study firstly reported associations of HIF-1 α , Flt-1 and VEGF with psoriasis via QHD intervention. These findings indicate QHD inhibits angiogenesis both in cultured cells and in mice. HIF-1 α /Flt-1/VEGF signaling is critical for angiogenesis, even in psoriasis. The TCM preparation QHD could inspire a rational development of new therapeutic options for clinical psoriasis. However, the molecular mechanism behind QHD's effect on the HIF-1 α /Flt-1/VEGF pathway is unclear, which deserves further investigation.

Declarations

Ethics approval

All animal studies were approved by the ethics committee of the Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine and the principles of laboratory animal care were followed in all animal experiments. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

The authors declare that they consent for publication.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Wen Li, Xiang He

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Provision of study materials or patients: Wen Li, Jiong Zhu

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Manuscript writing: All authors

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Figures

Figure 1

QHD inhibited proliferation of HUVECs cells and depressed the information of capillary tube in HUVECs cells. A: QHD reduces the viability of HUVECs cells. The cells were incubated with QHD at 0, 100, 200, 400, 800, 1600, 3200 and 6400 μ g/ ml for 48 h. The viable cells were determined using CCK-8 assay. Data presented were means $\pm$ SD of three independent experiments. $*P < 0.05$ compared with the control group. B: The angiogenesis capability of HUVECs cell in vitro is affected by QHD. HUVECs cells were treated with QHD at 20, 200 and 400 μ g/ml for 28h, the constitution of QHD was significantly decreased by QHD compared with that of untreated cells and MTX treated cells($*P < 0.05$)

Figure 2

QHD suppressed migration of HUVECs in vitro. A: The migrated cells towards the lower membranes. The migrated cells towards the lower membranes were stained with 0.1% crystal violet. Representative photomicrographs of migrated cells in control, QHD-L, QHD-M, QHD-H and MTX groups. (magnification 10 $\times$). QHD can significantly suppressed the migration of HUVECs. $P < .005$ vs. control and MTX groups. B: The wound scratch assay at 0, 12 and 24 h was recorded. The distance of migrated cells travelled in each treatment group was shown. The area of gap was of gap quantified at 0 h and 12 h in different groups. Calculated formula: the area of gap (%) = 1- area of 12 h/area of 0 h. QHD can significantly inhibit the migration capacity of HUVECs. $P < .005$ vs. control and MTX groups.

Figure 3

The Dorsal Skin Images of BALB/c Mouse Psoriasis Models Induced by Imiquimod. Psoriasis-like skin inflammation was induced to the back skin of the mice characterized by erythema and scaling.

QHD reduced angiogenesis was investigated through the dorsal skin images of BALB/c mouse psoriasis models induced by imiquimod. Statistical difference was analyzed by Student's t-test. $*P<0.05$ compared with that of control group and MTX group.

Figure 4

QHD impacts angiogenesis through HIF-1α/Flt-1/VEGF signal pathway in imiquimod (IMQ)-induced mouse model. A-B: The expression levels and expression site in cells of HIF-1α and Flt-1 in IMQ-induced mouse model by immunofluorescence. C: The protein expression of HIF-1α, Flt-1 and VEGF in IMQ-induced mouse model and negative control groups. D-F: HIF-1α, Flt-1 and VEGF mRNA levels were normalized to β-actin. The HIF-1α, Flt-1 and VEGF relative genes expression levels were calculated using the 2-ΔΔCT method. $P < .05$ was considered to be statistically significant. Graph shows mean $\pm$ SD. * $p < .05$, ** $p < .01$, *** $p < .05$.