図2　$CD11b^{-}$ IgA^{+} 細胞に比べ$CD11b^{+}$ IgA^{+}細胞で高発現している遺伝子オントロジー

GO ACCESSION	GO Term	p-value	corrected p-value	Count in Selecti	% Count in Selection	Count in Total	% Count in Total
GO 0044427	chromosomal part	3.91E-20	2.36E-15	144	4.123711	351	1.9954519
GO 0000279	M phase	2.31E-19	3.48E-15	123	3.5223367	286	1.6259239
GO 0022402	cell cycle process	1.55E-19	3.48E-15	160	4.5819016	411	2.3365548
GO 0022403	cell cycle phase	1.88E-19	3.48E-15	139	3.980527	339	1.9272314
GO 0007049	cell cycle	5.62E-19	6.77E-15	234	6.7010307	689	3.9169984
GO 0005694	chromosome	3.25E-18	3.26E-14	163	4.6678123	433	2.4616258
GO 0000278	mitotic cell cycle	2.83E-17	2.44E-13	110	3.1500573	257	1.4610574
GO 0000087	M phase of mitotic cell cycle	3.90E-16	2.93E-12	89	2.5486827	196	1.1142695
GO 0006996	organelle organization	1.37E-15	9.18E-12	332	9.507445	1133	6.4411597
GO 0048285	organelle fission	1.94E-15	1.17E-11	88	2.5200458	197	1.1199545

図2CD11b陽性とCD11b陰性IgA細胞を単離、精製しマイクロアレイ解析を行ったところ、CD11b陽性細胞で細胞周期に関わる分子の高発現が認められた。

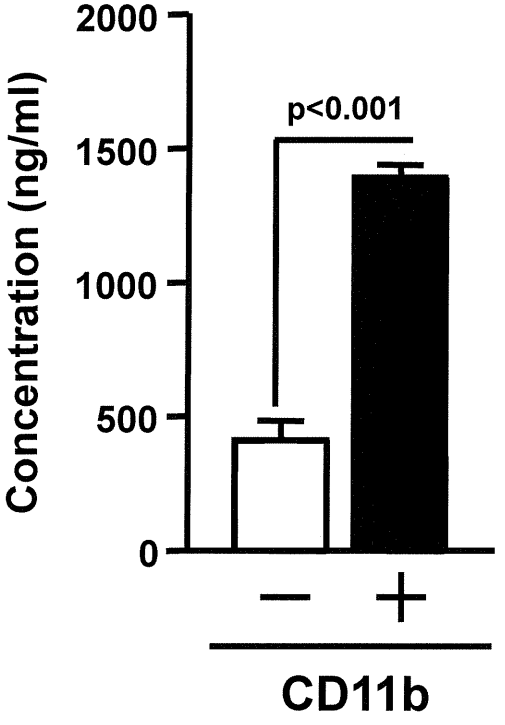

図3　CD11b陽性IgA細胞とCD11b陰性IgA細胞を単離、精製しin vitroで72時間培養した後の培養上清中のIgA量を測定したところ、CD11b陽性細胞で高いIgA産生が認められた。

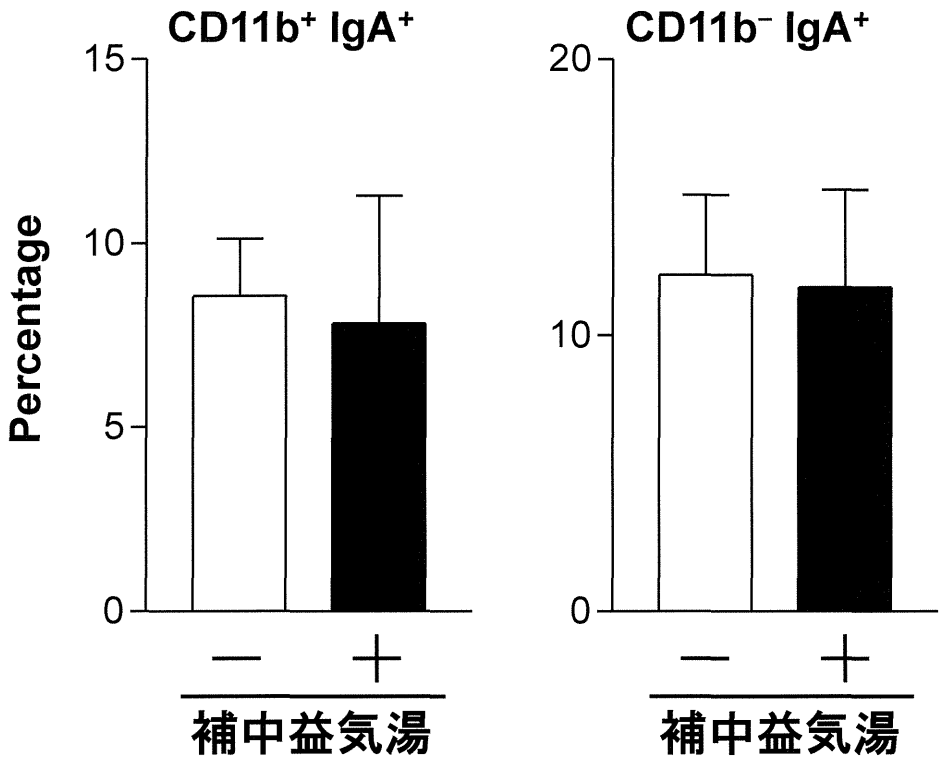

図4　コントロールマウス（□）と補中益気湯（■）を投与したマウスの腸管から単核球を回収し、CD11bとIgAでFACS解析したところ、補中益気湯によるCD11b陽性IgA細胞への影響は認められなかった。

厚生労働科学研究費補助金（医療技術実用化総合研究事業（臨床研究推進研究事業））
分担報告書

漢方薬ならびにその有効成分による マラリア感染免疫修飾機序の解明

研究分担者　平山謙二　長崎大学熱帯医学研究所免疫遺伝学分野

研究要旨

マラリア感染は媒介昆虫であるハマダラカによる感染型原虫スポロゾイトの皮膚からの侵入により起こる。原虫は血管内侵入後数分で肝臓のジヌソイドからクッパー細胞を通過し肝細胞内で増殖を開始する。増殖したメロゾイト原虫は5－6日で血液中に放出され今度は赤血球内に侵入しさらに増殖を繰り返す。脾臓が主な免疫応答の場と考えられるが、重症化すると重度の貧血や昏睡（脳マラリア）を発症し死に至ることがある。ここでは、脳マラリアのマウスモデルを用い、補中益気湯と十全大補湯の病態への影響を調べた。その結果、このモデルにおいて、いずれの漢方薬も神経症状を改善させ、特に十全大補湯では、脳マラリアでの死亡を減少させる効果のあることが示された。これらの薬剤投与による原虫血症の改善は見られなかったことから、この効果は病態と関係する免疫応答を修飾していることが推測された。

A. 研究目的

脳マラリアは熱帯熱マラリアによる死亡原因となるマラリアの最も重篤な臨床型である。昏睡を主たる症状とし、その病態の特徴は脳内の微小塞栓と出血である。ヒトでの病態生理については明らかではないが、マウスマラリアのある実験系を用いた脳マラリアモデルでは、脳内での病的なインターフェロンガンマ産生性CD8陽性T細胞の活性化が病態と強い関連があるとされている。免疫調節作用を有する漢方薬により、マラリア感染の際の免疫応答が修飾され、脳マラリアモデルの病態に変化が起こるか否かについて検討した。

B. 研究方法

1．6－8週令メスのC57Bl/6（B6）マウスに、マラリアのチャレンジ感染7日前から毎日と感染後5日間、40 mg/dayで蒸留水に溶解した十全大補湯（JTT）あるいは補中益気湯（HET）を経口投与した。陰性対照として蒸留水を同量飲ませたマウスを用意した。

２．投薬7日目にマウスの腹腔に*Plasmodium berghei ANKA*感染赤血球10万個を感染させた。感染後18日間にわたって原虫血症、体重変化、神経症状スコア、昏睡の有無、生存率を観察した。血液中のリンパ球分画およびインターフェロンガンマのレベルのモニターも行った。

（倫理面への配慮）

動物実験は長崎大学動物実験施設のガイド

ラインに則り行った。

C. 研究結果

2 回の繰り返し実験を行い、以下の結果が得られた。

1. JTT あるいは HET のいずれの内服投与群においても蒸留水投与群と原虫血症のレベルに相違は認められなかった。

2. 体重変化を観察すると感染 8 日目の体重減少が JTT 投与群で有意に抑制されていることが示された。また重篤度を示す皮膚反応でも、投与群がほぼ正常であるのに対し、蒸留水投与群ではほぼすべてが毛の逆立ちやふくらみを示し重篤度のスコアでも JTT および HET で有意差を示した。

3. 神経症状を歩き方の異常、運動麻痺、痙攣、昏睡をスコア化して比較すると重篤度と同様、蒸留水群と JTT 群の間で有意差をみとめた。

4. 感染後 8－10 日前後で起こる脳マラリアによる死亡を免れ、生き残ったマウスの生存率を比較したところ、蒸留水群に比較し多く生存する傾向があることが分かった。Kaplan Mayer 法による有意差については現在検討中である。

D. 考察

現在種々のタイプのマラリアワクチンが開発されているが、その中には経鼻や経口投与による伝播阻止ワクチンも含まれている。しかし粘膜免疫をねらったワクチンのマラリアでの有効性については異論も多い。もっとも大きな問題はマラリア感染の際の免疫応答が非常に複雑でいまだに、ワクチンが一体どのような免疫を付与すべきなのかが明らかでないことである。ここでは、脳マラリアというマラリアが引き起こす最も重篤な合併症のモデルに対する漢方薬の影響を見ることにより、病害制御に有効な免疫応答とは何かを探ろうと試みた。未熟なデータではあるが、病態を改善する明白なエビデンスを認めたことで、今後有効性の標的となる免疫応答についてさらに詳細な解析が必要になったと考える。

E. 結論

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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2)Men TT, Huy NT, Trang DT, Shuaibu MN, Hirayama K, Kamei K. A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity. Malar J. 2012 Aug 9;11:272. doi: 10.1186/1475-2875-11-272.

3)Omar AH, Yasunami M, Yamazaki A, Shibata H, Ofori MF, Akanmori BD, Shuaibu MN, Kikuchi M, Hirayama K. Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study. Malar J. 2012 May 17;11(1):168

2. 学会発表

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兵庫医科大学（第８１回日本寄生虫学会大会　プログラム集・抄録集，pp75，2012）

H.　知的財産権の出願・登録状況

1.特許出願

該当事項なし

2.実用新案登録

該当事項なし

3.その他

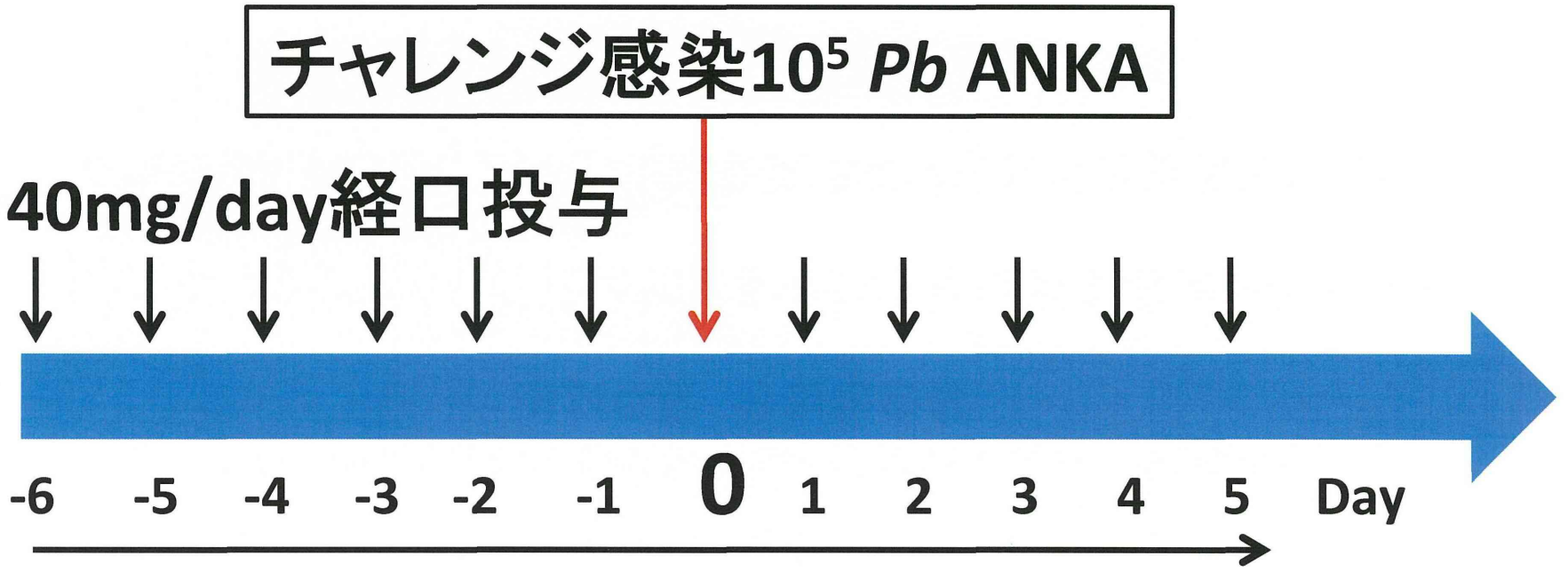
該当事項なし

十全大補湯(JTT)と補中益気湯(HET)の脳マラリアモデルマウスでの検討

厚生労働科学研究費補助金（創薬基盤推進研究事業）
H24年度　分担報告書

漢方薬ならびにその有効成分によるマラリア感染免疫修飾機序の解明

研究分担者　平山謙二　長崎大学熱帯医学研究所免疫遺伝学分野



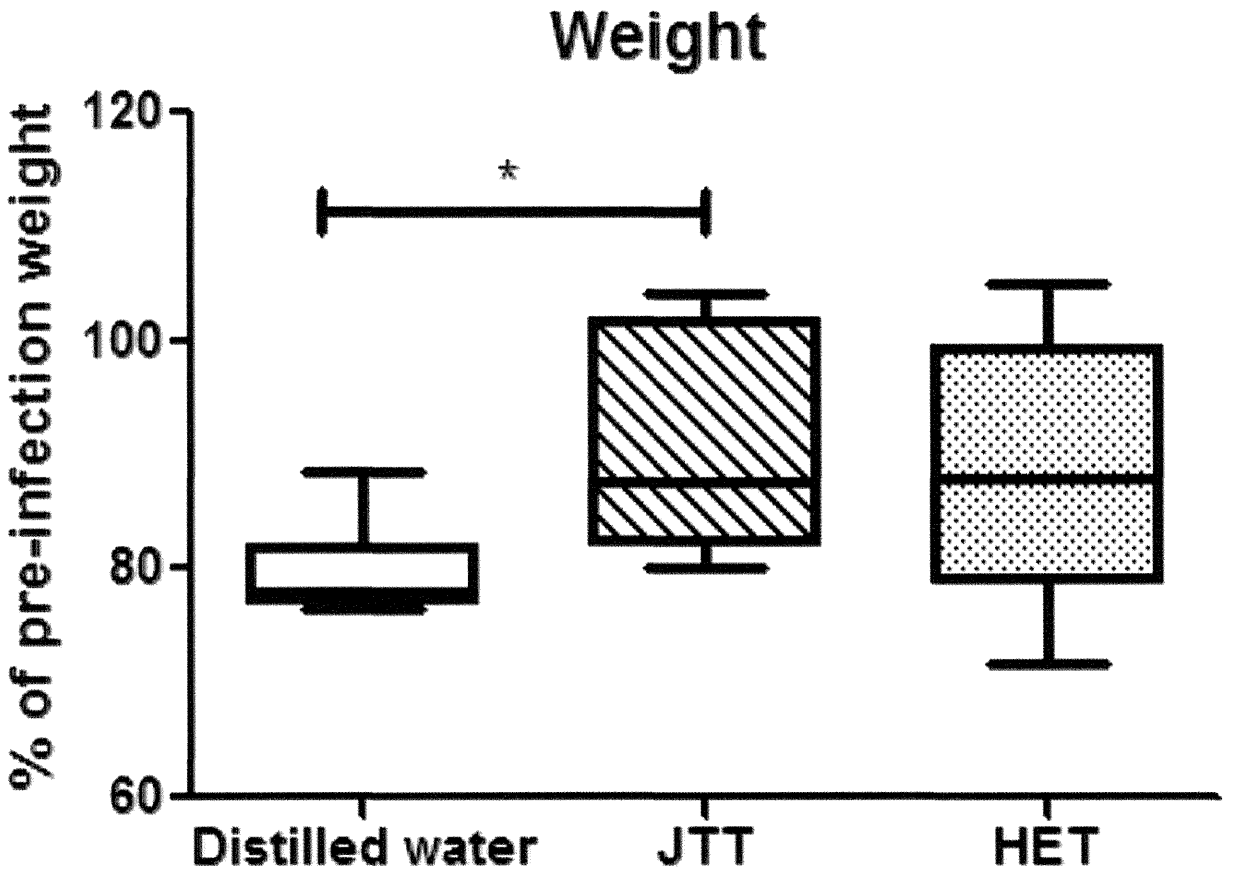
12 日間連続投与:3つのグループ

-蒸留水

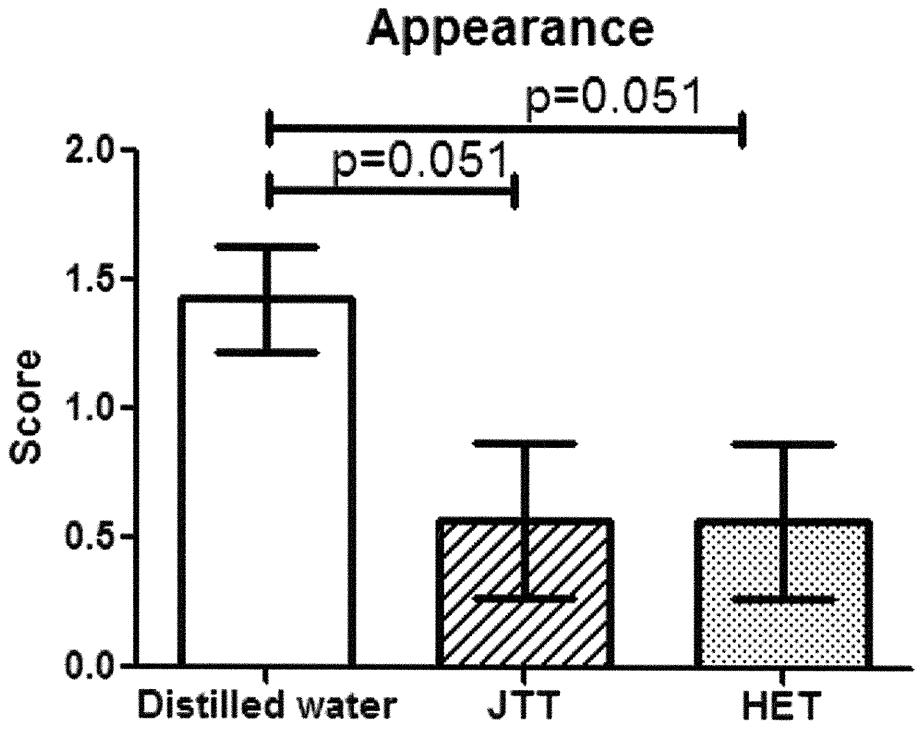
-十全大補湯 (JTT)

-補中益気湯 (HET)

感染8日目　体重変化

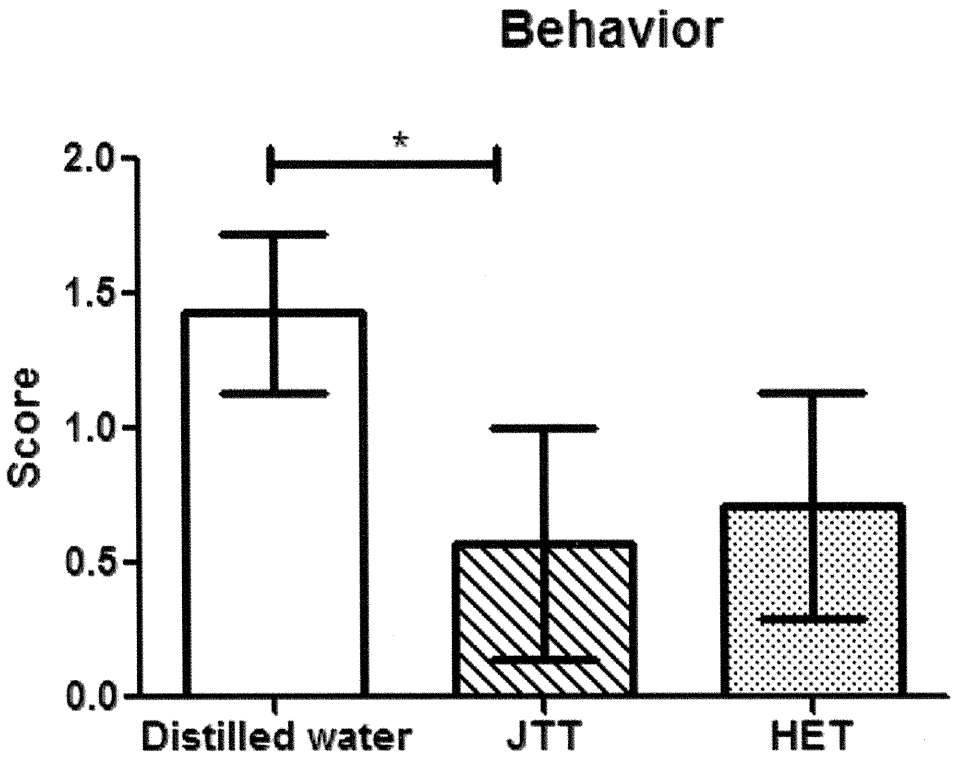

Weight
% of pre-infection weight
120
100
80
60
*
Distilled water
JTT
HET

感染8日目　重篤度変化



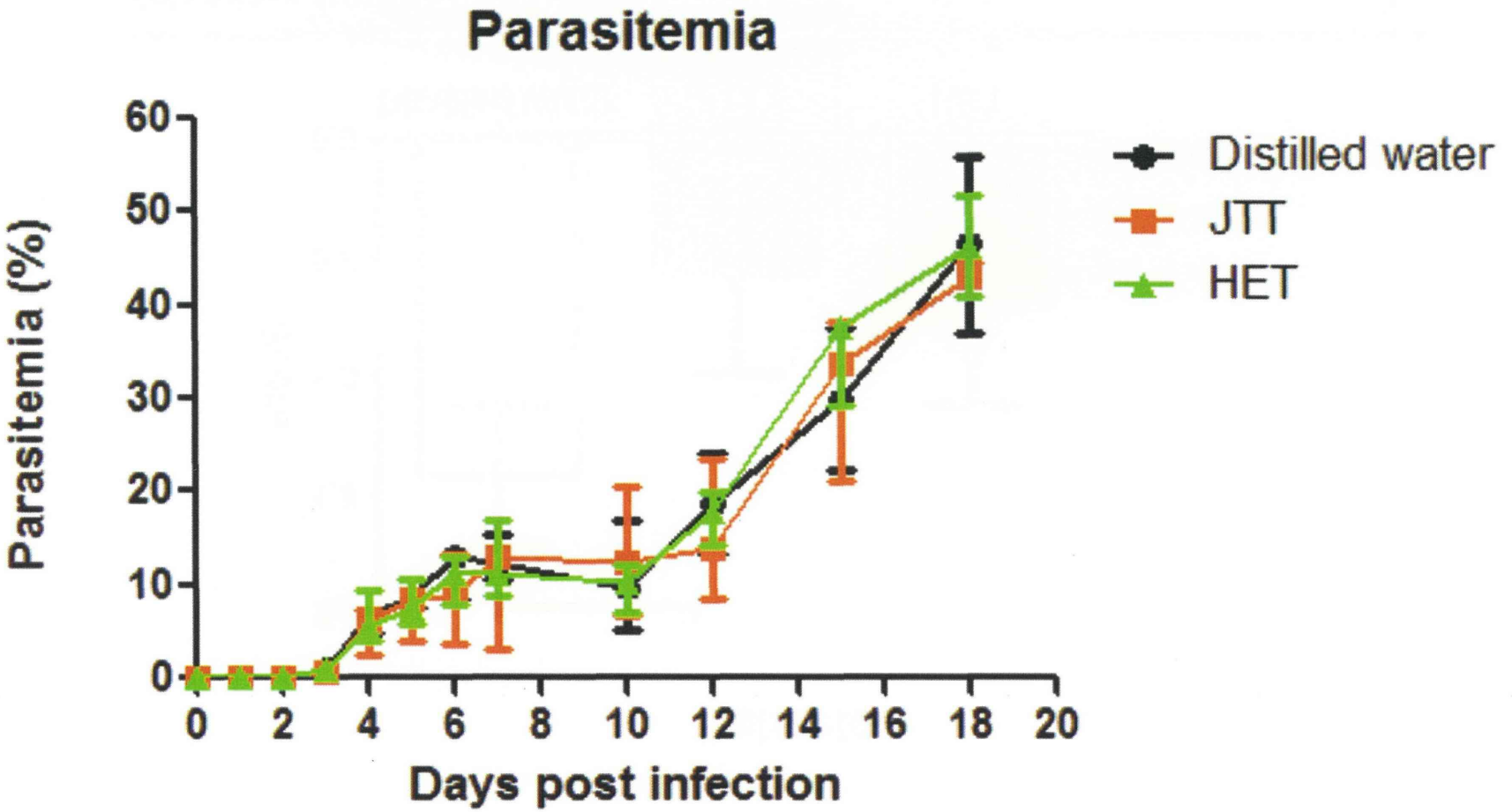
Appearance	Normal	0
	Coat ruffled	1
	Coat staring; panting	2

感染8日目　神経症状変化

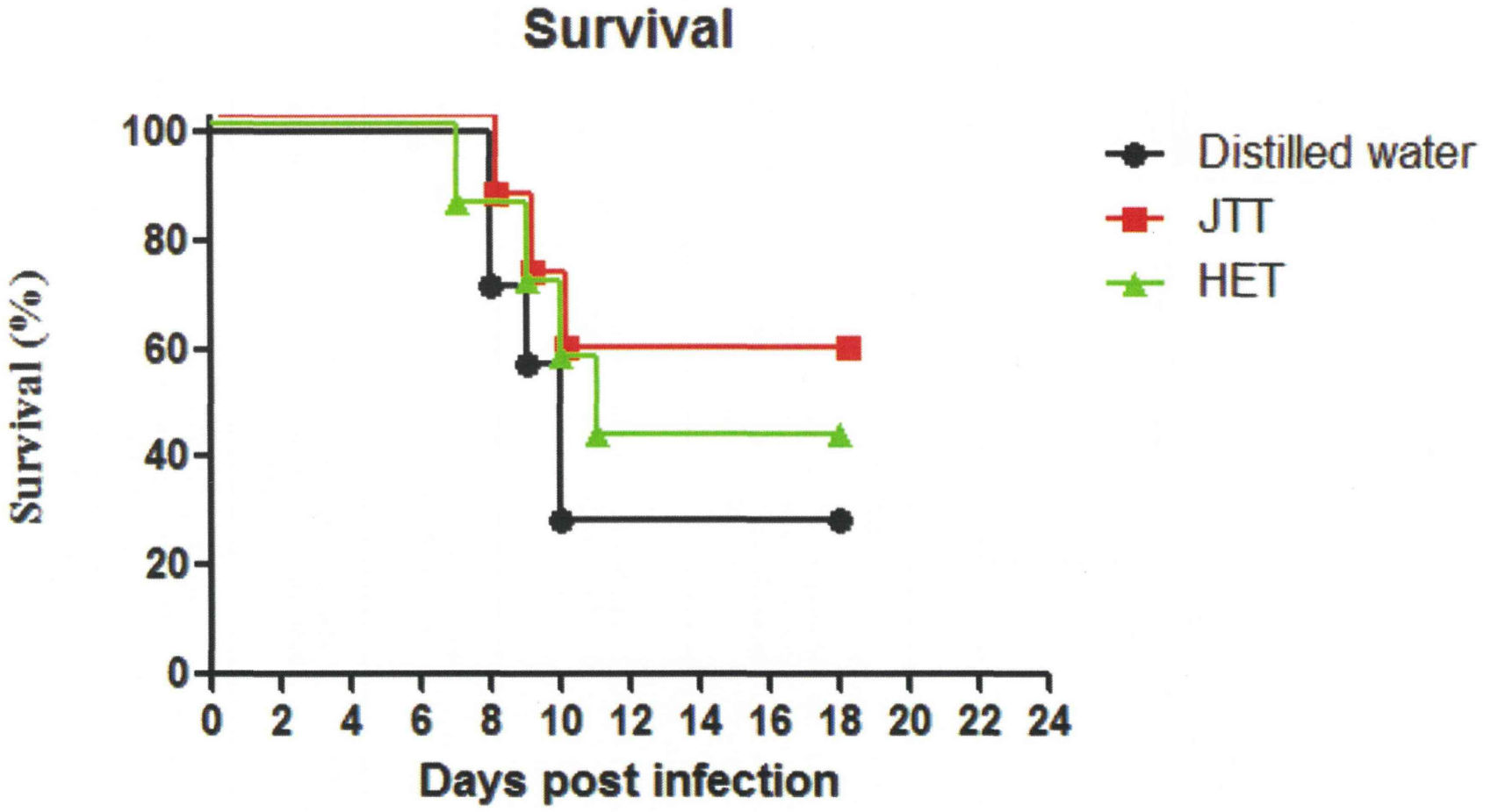


Behaviour (undisturbed)	Normal	0
	Hunched; wobbly gait	1
	Partial paralysis; immobile*†	2
	Convulsions; coma*	3

原虫血症の変化


Parasitemia
Parasitemia (%)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days post infection
Distilled water
JTT
HET

感染後18日間の生存曲線



研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
小泉桂一、犬嶌明子、大江未来広、柴原直利、済木育夫	トピックス：漢方薬のワクチンアジュバント効果	山田　正仁	最新医学	最新医学社	大阪	2013	869-873

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Orawin Prangsaengtong, Jun Yeon Park, Akiko Inujima, Yoshiko Igarashi, Naotoshi Shibahara and Keiichi	Enhancement of lymphangiogenesis *in vitro* via the regulations of HIF-1α expression and nuclear translocation by deoxyshikonin	Evid Based Complement Alternat Med		In press	2013
Sanphanya K, Wattanapitayakul SK, Prangsaengtong O, Jo M, Koizumi K, Shibahara N, Priprem A, Fokin VV, Vajragupta O	Synthesis and evaluation of 1-(substituted)-3-prop-2-ynylureas as antiangiogenic agents	Bioorg Med Chem Lett	22	3001-3005	2012
J. Kunisawa and H. Kiyono	Immune regulation and monitoring at the epithelial surface of the intestine	Drug Discovery Today	18	87-92	2013
J. Kunisawa, E. Hashimoto, I. Ishikawa, and H. Kiyono	A pivotal role of vitamin B9 in the maintenance of regulatory T cells in vitro and in vivo	PLoS One	7	e32097	2012
J. Kunisawa and H. Kiyono	Immunological function of sphingosine 1-phosphate in the intestine	Nutrients	4	154-166	2012
Men TT, Huy NT, Trang DT, Shuaibu MN, Hirayama K, Kamei K	A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity	Malar J	11	e272-	2012

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 148297, 11 pages
http://dx.doi.org/10.1155/2013/148297

Research Article

Enhancement of Lymphangiogenesis *In Vitro* via the Regulations of HIF-1α Expression and Nuclear Translocation by Deoxyshikonin

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Received 11 January 2013; Revised 19 March 2013; Accepted 21 March 2013

Academic Editor: Ken Yasukawa

The objectives of this study were to determine the effects of deoxyshikonin on lymphangiogenesis. Deoxyshikonin enhanced the ability of human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) to undergo time-dependent *in vitro* cord formation. Interestingly, an opposite result was observed in cells treated with shikonin. The increased cord formation ability following deoxyshikonin treatment correlated with increased VEGF-C mRNA expression to higher levels than seen for VEGF-A and VEGF-D mRNA expression. We also found that deoxyshikonin regulated cord formation of HMVEC-dLy by increasing the HIF-1α mRNA level, HIF-1α protein level, and the accumulation of HIF-1α in the nucleus. Knockdown of the HIF-1α gene by transfection with siHIF-1α decreased VEGF-C mRNA expression and cord formation ability in HMVEC-dLy. Deoxyshikonin treatment could not recover VEGF-C mRNA expression and cord formation ability in HIF-1α knockdown cells. This indicated that deoxyshikonin induction of VEGF-C mRNA expression and cord formation in HMVEC-dLy on Matrigel occurred mainly via HIF-1α regulation. We also found that deoxyshikonin promoted wound healing *in vitro* by the induction of HMVEC-dLy migration into the wound gap. This study describes a new effect of deoxyshikonin, namely, the promotion of cord formation by human endothelial cells via the regulation of HIF-1α. The findings suggest that deoxyshikonin may be a new drug candidate for wound healing and treatment of lymphatic diseases.

1. Introduction

Lymphangiogenesis is similar to angiogenesis and refers to the formation of lymphatic vessels from preexisting lymphatic vessels, which play an important role in tissue-fluid homeostasis, as a tissue drainage system, immunosurveillance, and absorption of dietary fat [1]. Dysfunction of lymphatic vessels leads to chronic edema and impairment of immune responses. In adult tissue, the induction of new lymphatic vessel growth also promotes inflammation, wound healing, and tumor metastasis to the lymph node [2]. The molecular mechanisms of angiogenesis and its treatment are already well known, whereas understanding of the functions and regulatory pathways of lymphangiogenesis and its treatment has been far less explored [1].

Vascular endothelial growth factors (VEGFs) are interesting inducers of lymphangiogenesis, because they are a highly specific mitogen for endothelial cells [3] and transcriptional factors; hypoxia-inducible factor-1 (HIF-1), which is composed of two subunits, HIF-1alpha (HIF-1α) and HIF-1beta (HIF-1β) [4, 5], can modulate VEGF gene expression [6–8]; however, the role of these regulators in the lymphangiogenesis process is poorly understood.

Shiunko is a typical Kampo drug ointment (a traditional botanic formula) used for the treatment of burns and wounds in Japan [9, 10]. Shiunko has been proved

to improve wound healing by promoting reepithelialization and granulation tissue formation, including angiogenesis [10]; however, there are on reports of the effect of shiunko on lymphangiogenesis. Lymphangiogenesis and angiogenesis are important processes in wound healing [11] and the efficacy of shiunko for the promotion of lymphangiogenesis and also angiogenesis in wound healing may be derived from the effect of components of this herbal medicine.

One of the components of shiunko is Lithospermi Radix (LR, the dried root of *Lithospermum erythrorhizon* Sieb. et Zucc, also called Zicao or Gromwell) that contains several compounds of shikonin and its derivatives, such as deoxyshikonin, acetylshikonin, isobutylshikonin, and others [12]; however, there is no report on shikonin and its derivatives that promote lymphangiogenesis or angiogenesis. In contrast, we found that shikonin and some derivatives have strongly shown to inhibit angiogenesis in *in vitro* and *in vivo* models [9] by suppressing VEGF production, proliferation, and the migration of endothelial cells [13]. These compounds also blocked integrin $\alpha v\beta 3$ expression and inhibited B16 melanoma- and tumor necrosis factor-alpha-induced angiogenesis in mice [9]. To find another shikonin derivative that may have an effect on angiogenesis and lymphangiogenesis, deoxyshikonin was examined in this study. Because traditional medicine for lymphangiogenesis treatment has not been explored widely, this study attempted to find a new mechanism of this compound for controlling lymphangiogenesis *in vitro*.

2. Materials and Methods

2.1. Materials. Deoxyshikonin was purchased from Tokyo Chemical Industry (TCI) (Tokyo, Japan). The compounds were dissolved in dimethylsulfoxide (DMSO) to make a stock solution. Matrigel was purchased from BD Biosciences (San Diego, CA, USA). HIF-1α siRNA and the antibodies against HIF-1α and PCNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal rabbit anti-mouse immunoglobulin/HRP, polyclonal goat anti-rabbit immunoglobulin/HRP, and polyclonal swine anti-rabbit immunoglobulins/FITC were purchased from Dako (Glostrup, Denmark). Dynabeads protein G was purchased from Invitrogen (Oslo, Norway). Vectashield mounting medium with DAPI was from Vector Laboratories, Inc. (Burlingame, CA, USA). Rhodamine phalloidin was obtained from Life Technologies (Carlsbad, CA, USA). The culture inserts were from Ibidi (Martinsried, Germany).

2.2. Endothelial Cells. Human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) and human dermal microvascular endothelial cells (HMVEC-d) were obtained from Takara Bio Inc. (Shiga, Japan). The cells were primary culture cells. Cells were cultured in Clonetics EGM-2 MV Bullet Kit (Takara Bio) in a humidified atmosphere (5% CO_2, 95% air). Cells were passaged upon reaching confluence with Trypsin-EDTA solution. To maintain normal growth, the primary cells from 5th to 15th passages were used in the study.

2.3. Proliferation Assay. Cell viability after treatment with various concentrations of deoxyshikonin and shikonin was assessed with a WST-8 cell proliferation assay kit (DOJINDO, Kumamoto, Japan). Cells were cultured in 96-well plates at 37°C. At the time of measurement, 10 μL WST-8 reagent was added to each well and the cells were cultured continuously for 2 h at 37°C in 5% CO_2. Absorbance was measured at 450 nm to determine cell viability as a percentage.

2.4. Cord Formation on Matrigel. Ninety-six-well plates were coated with 60 μL Matrigel (10 mg/mL) and allowed to polymerize at 37°C. Endothelial cells (8×10^3 cells/well) were seeded on the Matrigel and incubated at 37°C. At each time point, cells were fixed with a 4% paraformaldehyde and stained using Mayer's hematoxylin (Muto Pure Chemical, Tokyo, Japan). The cord network was photographed and cord length was measured using an Angiogenesis Image Analyzer (Kurabo, Osaka, Japan) [14].

2.5. Gene Expression Analysis by Real-Time PCR. Briefly, total RNA was extracted from cultured cells on Matrigel using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 0.5 μg of total RNA was reverse transcribed into cDNA using the Prime Script RT reagent kit (Perfect Real Time) (TaKaRa, Dalian, China). Real-time PCR analysis was performed using the LightCycler Nano System (Roche Diagnostics, Mannheim, Germany) using FastStart Essential DNA Green Master (Roche Diagnostics) according to the manufacturer's instructions. GAPDH was used as an internal control. The relative quantification of mRNA expression was calculated as a ratio of the target gene to GAPDH. The primer sequences were as follows: HIF-1α sense, 5′-TTTTTCAAGCAGTAGGAATTGGA-3′, and antisense, 5′-GTGATGTAGTAGCTGCATGATCG-3′; VEGF-C sense, 5′-TGCCAGCAACACTACCACAG-3′, and antisense, 5′-GTGATTATTCCACATGTAATTGGT-G-3′; VEGF-A sense, 5′-CCTCCGAAACCATGAACTTT-3′, and antisense, 5′-ATGATTCTGCCCTCCTCCTT-3′; VEGF-D sense, 5′-GGAGGAAAATCCACTTGCTG-3′, and antisense, 5′-GCAACGATCTTCGTCAAAC-3′; GAPDH sense, 5′-AGCCACATCGCTCAGACAC-3′, and antisense, 5′-GCCCAATACGACCAAATCC-3′.

2.6. Detection of HIF-1α and PCNA. To determine protein levels during cord formation on Matrigel, immunoprecipitation and Western blotting were performed as described previously [14]. Cells cultured on Matrigel were washed with PBS and incubated with whole cell lysis buffer (25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/mL aprotinin, and 10 μg/mL leupeptin). The cells including Matrigel were then scrubbed. The collected samples were vigorously vortexed, centrifuged at 14,000 rpm for 10 min, and the supernatant was collected. Immunoprecipitation was carried out by incubating the lysate with HIF-1α primary antibody (Santa Cruz Biotechnology) for 16 h at 4°C, followed by 12 h incubation with Dynabeads

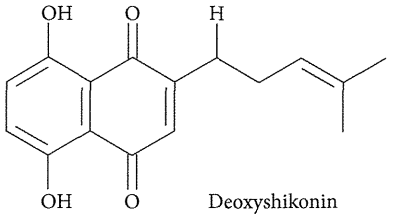

FIGURE 1: Chemical structure of deoxyshikonin.

protein G (Invitrogen). The immunoprecipitates were washed with lysis buffer, resuspended in loading buffer, boiled for 3 min, subjected to SDS-PAGE on 7.5% polyacrylamide gels, and transferred to a PVDF membrane. The primary antibody used was for HIF-1α and the secondary was polyclonal goat anti-rabbit immunoglobulin/HRP. The bands were detected using an immunochemiluminescence method. PCNA was used as the loading control.

2.7. Immunofluorescence Microscopy. Cells were seeded onto a cover slip coated with Matrigel (10 mg/mL). Cells were incubated with or without deoxyshikonin-containing medium. At the time of the experiment, the attached cells were washed with PBS, fixed with 4% paraformaldehyde (10 min), and washed and permeabilized (5 min) with 0.1% Triton X-100 in PBS. Samples were blocked with 1% BSA in PBS followed by incubation for 30 min with HIF-1α rabbit polyclonal primary antibody (Santa Cruz Biotechnology). After incubation with primary antibody, cells were washed in 0.2% Triton X-100 in PBS and then incubated with polyclonal swine anti-rabbit immunoglobulins/FITC (Dako) as a secondary antibody and Rhodamine phalloidin (Life Technologies) for 20 min. After washing, Vectashield mounting medium for DAPI staining (Vector, Burlingame, CA, USA) was added to the cells. Florescence images were captured using a Leica TCS SP5 microscope.

2.8. siRNA Transfection. Proliferating HMVEC-dLy was transfected with control siRNA or siRNA against HIF-1α (Santa Cruz Biotechnology) at a final concentration of 6 nM using Lipofectamine RNAiMAX reagents (Invitrogen). After transfection, the cells were grown for 18 h at 37°C in 5% CO_2 and trypsinized. The transfected cells combined with or without deoxyshikonin were seeded on Matrigel-coated dishes. At each time point, cells were employed for real-time PCR and cord formation assays.

2.9. Wound-Healing Assay. To investigate the potential wound-healing ability with deoxyshikonin treatment, a modified scratch assay was performed, creating gaps of precisely defined width. Culture inserts from Ibidi (Martinsried, Germany) were used in this study. This insert creates a cell-free gap (approximately 500–600 μm) [15]. Seventy microliters of cell suspension (1.8×10^5 cells/mL) were added to each well of the Ibidi culture insert. Cells were incubated at 37°C for 48 h until the cells were confluent and then the culture inserts were removed to create the gap and to allow cell migration to fill it over time. Cell migration into the gap was monitored by inverted microscopy and photographed at each time point. The distance between one side of the gap and the other can be measured by comparing the image from time 0 h to the last time point at 24 h. The distance between each gap closer was measured using Leica LAS EZ software and then calculated as the migration distance (mm).

2.10. Statistical Analysis. Statistical analysis was performed using Dunnett's method. $P < 0.05$ was considered to be significant.

3. Results

3.1. Deoxyshikonin Enhanced Cord Formation of HMVEC-dLy and HMVEC-d on Matrigel. Shikonin and some shikonin derivatives have been reported to inhibit angiogenesis [13, 16]. To find new effect of compounds that affects lymphangiogenesis and angiogenesis, deoxyshikonin was selected for use in the present study. The nontoxic dose of 0.8 μM deoxyshikonin was used to see the effect on cord formation ability of human lymphatic endothelial cells (HMVEC-dLy) and human dermal microvascular endothelial cells (HMVEC-d) (Figure 2). The cells underwent the cord formation assay and were photographed (Figures 2(a) and 2(c)) at 2 to 6 h after seeding on Matrigel. Cord length was measured by an Angiogenesis Image Analyzer and plotted as a percentage (Figures 2(b) and 2(d)). Deoxyshikonin significantly promoted cord formation ability by 64% and 28% from the control in HMVEC-dLy and HMVEC-d at 6 h of incubation, respectively (Figures 2(b) and 2(c)). This is a newly discovered effect of deoxyshikonin, which promoted to lymphangiogenesis and angiogenesis in an *in vitro* model and showed the opposite effect to shikonin (data not shown).

Because knowledge about the mechanism of lymphangiogenesis and treatment with natural compounds has not been explored sufficiently, we decided to further confirm the possible mechanism of this natural compound, deoxyshikonin, on lymphangiogenesis.

The time that showed a significant change in cord formation networks, 6 h of deoxyshikonin treatment in HMVEC-dLy, was chosen for use in further experiments.

3.2. Deoxyshikonin Dominantly Increased VEGF-C mRNA Level in HMVEC-dLy While Forming Cords on Matrigel. Endothelial cells are the target of VEGF-C, -A, and -D in lymphangiogenesis induction [4]. In addition, endothelial cells themselves can express VEGF mRNA and protein levels after stimulation [17]. The cord formation ability of HMVEC-dLy, which was enhanced by deoxyshikonin (Figures 2(a) and 2(b)), may occur as a result of the increase of VEGFs.

We further examined the effect of deoxyshikonin on the expression of VEGF-C, VEGF-A, and VEGF-D mRNA during cord formation of HMVEC-dLy. Real-time PCR was used to determine transcription levels of these genes.

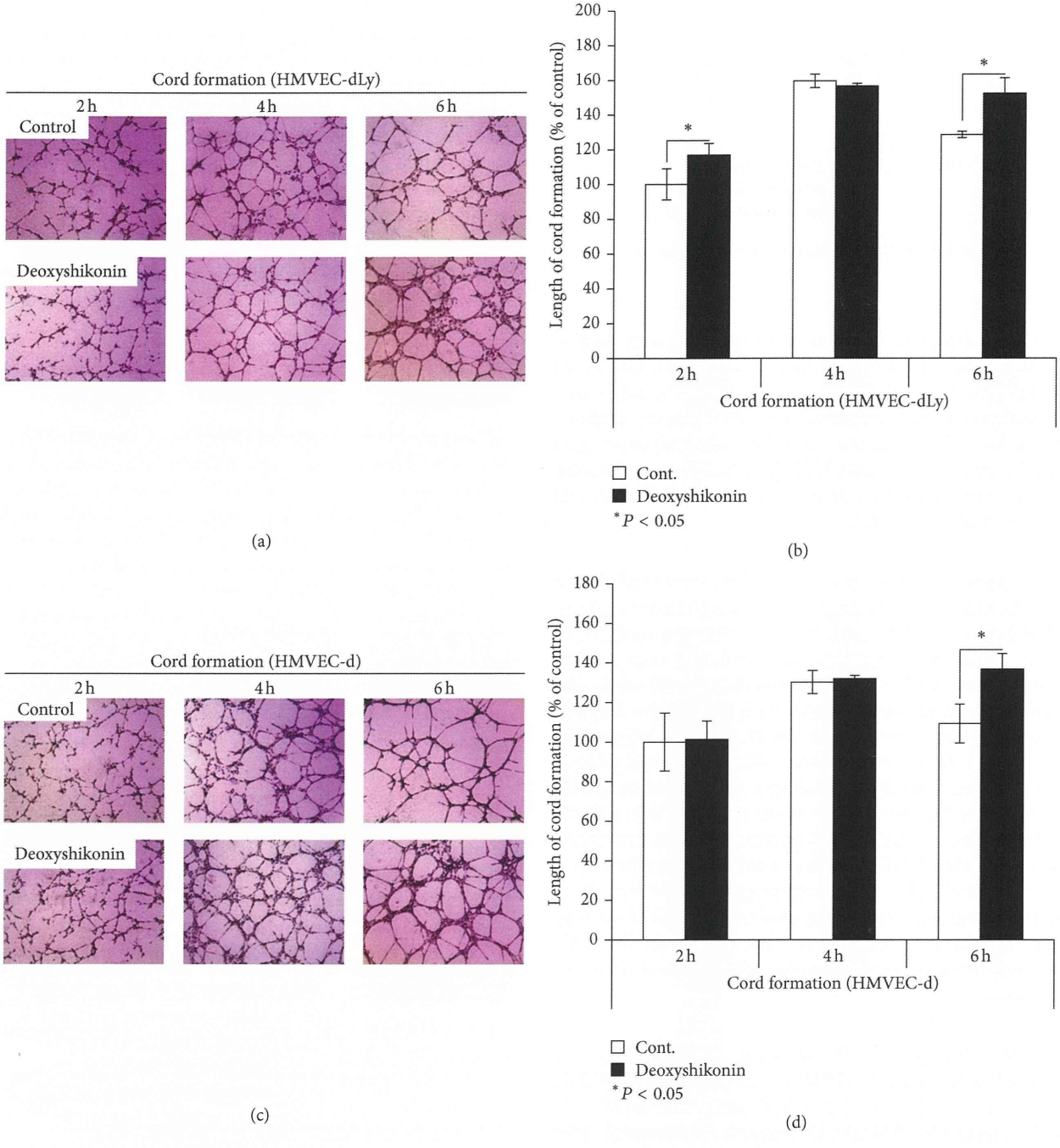

FIGURE 2: Effects of deoxyshikonin on cord formation of HMVEC-dLy and HMVEC-d on Matrigel. (a), (c) Photographs of cord formation of HMVEC-dLy and HMVEC-d on Matrigel after incubation with or without 0.8 μM deoxyshikonin at 2 to 6 h (at ×400 magnification). (b), (d) The relative length of cords was measured using an Angiogenesis Image Analyzer. Data are the mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

Endothelial cells were seeded on Matrigel and incubated with 0.8 μM deoxyshikonin for 6 h. The mRNA was collected and subjected to real-time PCR (Figure 3(b)). The cord formation assay was also performed for comparison at the same time of incubation (Figure 3(a)). The results showed that deoxyshikonin significantly increased mRNA expression levels of VEGF-C and VEGF-A in HMVEC-dLy by 0.42-fold and 0.32-fold when compared with their control, respectively (Figure 3(b)). These increases also correlated with the increase of cord formation of endothelial cells at the same time of treatment (Figure 3(a)); however, VEGF-D mRNA levels were expressed at very low levels (Figure 3(b)). This indicated that deoxyshikonin-induced cord formation networks in HMVEC-dLy were involved in the induction of

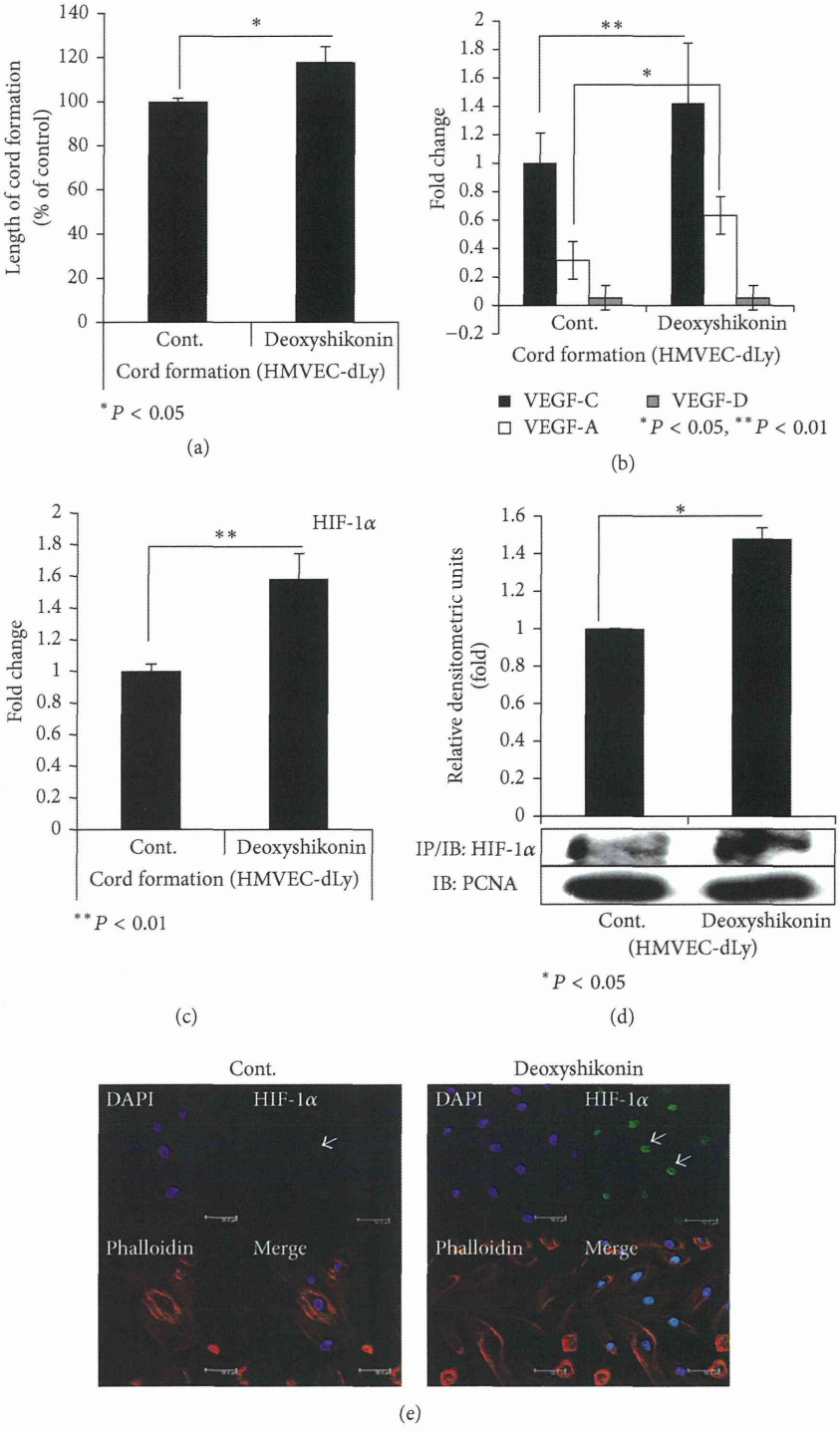

FIGURE 3: Effect of deoxyshikonin on VEGF-C, -A, and -D mRNA levels and regulation of HIF-1α during cord formation of HMVEC-dLy on Matrigel. Cells were exposed with or without 0.8 μM deoxyshikonin for 6 h and then underwent experiments. (a) The relative length of cords was measured using an Angiogenesis Image Analyzer. (b), (c) VEGF-C, -A, and -D and also HIF-1α mRNA levels were detected by real-time PCR. (d) The HIF-1α protein level was determined by immunoprecipitation and Western blotting. The results were analyzed by scanning and Scion Image software. (e) HIF-1α nuclear translocation as determined by immunofluorescence microscopy. Similar results were obtained in three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with their control.

VEGF-C and VEGF-A mRNA levels, which had a greater potential effect on the increase of the VEGF-C mRNA level than the VEGF-A mRNA level (Figure 3(b)).

VEGF-C is the main vascular endothelial growth factor important for lymphangiogenesis [18, 19]. As the next step, we studied the possible mechanisms of deoxyshikonin induction in lymphangiogenesis *in vitro* by using HMVEC-dLy.

3.3. Deoxyshikonin Regulates HIF-1α at Transcriptional, Post-transcriptional, and Functional Levels in HMVEC-dLy during Cord Formation on Matrigel. HIF-1α plays a certain role in lymphangiogenesis by closely correlating with lymphatic expression of VEGF-C in cancers, wound healing, and inflammation [7, 20, 21], and we found that deoxyshikonin upregulated the VEGF-C mRNA level during lymphangiogenesis *in vitro* (Figure 3(b)). In addition, the function of HIF-1 can be regulated by several stimuli under normoxic conditions [4, 5]. Deoxyshikonin, which promoted cord formation of HMVEC-dLy in this study, may correlate with VEGF-C mRNA expression and HIF-1α regulation.

To determine the effect of deoxyshikonin on HIF-1α regulation, real-time PCR, immunoprecipitation/Western blotting, and immunofluorescence microscopy were performed to see the expression of HIF-1α mRNA, HIF-1α protein, and also the activation of HIF-1α, respectively. HMVEC-dLy were seeded on Matrigel, cultured with or without 0.8 μM deoxyshikonin-containing medium, and left to form cord networks for 6 h. Then, mRNA and protein were collected to measure mRNA and protein expression levels. Figure 3(c) shows that the HIF-1α mRNA level was increased 0.58-fold that of the control (Figure 3(c)) and the HIF-1α protein level also significantly increased after deoxyshikonin treatment (Figure 3(d)). The nuclear translocation of HIF-1α was also determined (Figure 3(e)). Cells were seeded on Matrigel-coated slides and then treated with or without 0.8 μM deoxyshikonin for 6 h. Immunofluorescence microscopy was performed and photographed (Figure 3(e)). HIF-1α is a cytoplasmic protein. During activation, HIF-1α will dimerize with HIF-1β and then translocate to the nucleus to give the active transcription factor of HIF-1. In our results, HMVEC-dLy treated with deoxyshikonin showed the accumulation of HIF-1α inside the nucleus, which indicated the activation of HIF-1α transcription factor. These results conclude that deoxyshikonin induced cord formation of HMVEC-dLy and was involved in the regulation of HIF-1α at the transcriptional, posttranscriptional, and functional levels.

3.4. Deoxyshikonin-Induced VEGF-C mRNA Expression and Cord Formation of HMVEC-dLy via HIF-1α Regulation. To see whether HIF-1α controls VEGF-C mRNA expression during cord formation of deoxyshikonin-treated cells, siHIF-1α transfection was used in this study. siCont- and siHIF-1α-transfected cells were seeded on Matrigel and incubated with or without 0.8 μM deoxyshikonin for 2–6 h. At each time point, cells underwent a cord formation assay (Figure 4(c)) and mRNA was collected to perform real-time PCR (Figures 4(a) and 4(b)). The results showed that HMVEC-dLy, which was transfected with siHIF-1α, successfully suppressed HIF-1α mRNA expression throughout the experiment (Figure 4(a)). During incubation, in the absence of deoxyshikonin, VEGF-C mRNA expression was significantly decreased in HIF-1α knockdown cells, by 0.25-fold, 0.5-fold, and 0.2-fold at 2, 4, and 6 h, respectively (Figure 4(b)) and these decreases also correlated with the significant decrease in the length of cord formation by the HIF-1α knockdown cells, by 38%, 56%, and 46% at 2, 4, and 6 h, respectively, (Figure 4(c)). These results indicated that HIF-1α controlled VEGF-C mRNA expression and cord formation ability in HMVED-dLy. In addition, deoxyshikonin treatment in siCont-transfected cells significantly increased HIF-1α and VEGF-C mRNA expression at 6 h (Figures 4(a) and 4(b)) when compared with their control groups. These increases also correlated with the significantly increased cord formation at the same incubation time (6 h) (Figure 4(c)). Interestingly, deoxyshikonin treatment of HIF-1α knockdown cells did not restore VEGF-C mRNA expression (Figure 4(b)) and cord formation (Figure 4(c)) to the control levels but only slightly increased their levels when compared to untreated HIF-1α knockdown cells.

These results indicated that deoxyshikonin promoted VEGF-C mRNA expression and the cord formation ability of HMVEC-dLy, mainly via HIF-1α-dependent regulation.

3.5. Deoxyshikonin Promoted Wound Healing In Vitro. We therefore succeeded in proving the mechanism of deoxyshikonin on the cord formation of HMVEC-dLy, which was involved in the regulation of HIF-1α and VEGF-C mRNA expression. As the next step, we assessed the potential of deoxyshikonin-induced lymphangiogenesis for clinical applications such as wound-healing treatment, because the promotion of lymphangiogenesis can improve wound-healing [22]. We performed a wound healing assay using culture inserts from Ibidi to create a cell-free gap and measured the gap distance (migration distance) using Leica LAS EZ software. The result showed that incubating the cells with 0.8 μM deoxyshikonin promoted the migration of HMVEC-dLy by significantly inducing cell filling of the gap when compared to the control at 24 h of incubation (Figures 5(a) and 5(b)). Some of the effect of deoxyshikonin on apparent cell migration might be a contribution from cell proliferation. The proliferation assay (Figure 5(c)) confirmed that at a deoxyshikonin concentration of 0.8 μM, the cells filling the gap of the wound did not arise from cell proliferation. However, at higher concentrations (1.6 and 3 μM), deoxyshikonin significantly induced proliferation of HMVEC-dLy. This result indicated that deoxyshikonin could be used to improve wound healing by inducting lymphatic endothelial cell migration and lymphangiogenesis.

4. Discussion

In this study, we used primary endothelial cells, human dermal lymphatic microvascular endothelial cells (HMVEC-dLy), to investigate a new effect of deoxyshikonin (Figure 1), on lymphangiogenesis *in vitro* by comparing them with