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원저

Puerariae Radix Induces Angiogenesis *in vitro* and *in vivo*

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Abstract

Background & Objective : Angiogenesis consists of the proliferation, migration, and differentiation of endothelial cells, and angiogenic factors and matrix protein interactions modulate this process. The aim of this study was to determine whether Puerariae radix could induce angiogenic activity in human umbilical vein endothelial cells (HUVECs).

Methods : The angiogenic activity of Puerariae radix were evaluated by using BrdU assay, chemotactic migration assay, tube formation assay, measurement of bFGF in HUVECs, and Matrigel plug assay in mice.

Results : Puerariae radix significantly increased HUVECs proliferation in a dose-dependent manner. In addition, Puerariae radix increased migration and tube-like formation in HUVECs. Interestingly,the expression of basic fibroblast growth factor (bFGF), an angiogenesis-stimulating growth factor, was dose-dependently increased by Puerariae radix. The angiogenic activity of Puerariae radix was confirmed using an in vivo Matrigel angiogenesis model, showing promotion of blood vessel formation.

Conclusion : Puerariae radix significantly induces angiogenesis in vitro and in vivo. These results suggest that Puerariae radix is a potent angiogenic agent, and a promising drug, for the induction of neovascularization.

Key words : Puerariae radix Angiogenesis, Proliferation, Migration, Tube formation, bFGF, Matrigel plug assay

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Ⅰ. Introduction

Angiogenesis is the process of forming new capillary blood vessels from preexisting vasculature, leading to neovascularization, and is a tightly controlled process that rarely occurs under normal conditions, except in the healing of wounds and the development of the embryo and the corpus luteum[1]. Generally, initiation of blood vessel formation involves several steps beginning with enzymatic degradation of the associated basement membrane. Vascular endothelial cells then migrate into the stromal space, proliferate and align. The cells form tubular structures, undergo significant remodeling, and finally reestablish a new basement membrane[2-4]. In addition, angiogenesis requires proper stimulation by angiogenic factors such as platelet-derived growth factor, bone morphogenic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF, FGF-2)[4-6].

bFGF has pleiotropic roles in many cell types and tissues; it is a mitogenic, angiogenic and survival factor involved in cell migration, cell differentiation and in a variety of developmental processes[7-8]. In addition, recent studies have shown that the use of bFGF as a therapeutic agent for the treatment of wound healing[9], ischemic cardiovascular disease[10-11], and bone fracture healing[12] is promising, and clinical trials are in progress. However, a potential alternative strategy may be to use drugs with angiogenic activity that are available in oral formulations and that are currently administered to patients for treatment of different pathologies[13].

Puerariae radix (PR), the root of Pueraria lobata Ohwi, a wild creeper leguminous plant, is one of the earliest and most important crude herbs used in oriental medicine. The clinical use for various diseases in internal medicine, surgery, pediatrics, dermatology and E.N.T. has also proved effective. The efficacy for arrhythmia is obvious[14]. Recently, It has been demonstrated that Pueraria lobata Ohwi extract and saponin from Puerariae radix preventive effects on liver injury of primary hepatocyte cultures[15]. Also, Pueraria lobata extract and its main compound reported several biological activities including anti-oxidative activity possess in rat liver cells[16]. It has been reported that Puerariae radix prevents bone loss by growth hormone release in ovariectomized rat[17]. However, much more insight into the pharmacological functions and mechanisms of Puerariae radix are needed, especially as there is no clear experimental evidence supporting its induces neovascularization for treatment such as wound healing and cardiovascular diseases. In this study, we attempted to identify and characterize whether Puerariae radix could induces angiogenesis in vitro and in vivo.

Ⅱ. Materials and Methods

1. Preparation of Puerariae radix extract

The root of Puerariae radix was extracted at room temperature in 70% (v/v) ethanol in water for 24 h. The extract was then filtered and concentrated under low pressure using a rotary vacuum evaporator (Eyela, Japan). The residue was lyophilized in a freeze dryer, and stored at 20 C. This powder, dissolved in dimethylsulfoxide (DMSO), was used for experiments with the final concentration of DMSO in the culture medium adjusted to below 0.5%.

2. Isolation and culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained by an established method from freshly delivered umbilical cords. In brief, human umbilical cord veins were cannulated and flushed with cold phosphate buffered saline (PBS) containing 0.2% glucose to remove blood and then filtered with 0.2% type II collagenase (SigmaAldrich Co., MO. USA) in PBS for 10 min at 37 C. After pelleting and resuspending the cells, they were plated in a 75 cm2 tissue culture flask coated with 0.1% gelatin, cultured with EGM-2 complete medium (Cambrex, MD, USA), and incubated at 37 C in 5% CO2. Once confluent, the cells were detached using a trypsin-EDTA solution and used in experiments from the third to sixth passages.

3. Cell proliferation assay.

HUVECs were plated at a density of 5 103 cells/well in EGMTM-2 medium in 96-well plates. After 24 h, the medium was removed and replaced with EBM (Cambrex Inc., MD, USA) plus 2% FBS, and 3 units/ml of heparin (control medium). Cells were treated with 0.01, 0.1, 1, 10, 100 g/ml of Puerariae radix or 5 ng/ml of bFGF (R&D Systems Inc., MN, USA). After 72 h incubation, 10 $\mu\ell$ of BrdU were added to each well, and the plates were incubated for a further 6 h at 37 C. Cells were fixed, and anti-BrdU-POD was then added and detected by the TMB substrate reaction. This reaction was quantified using an ELISA reader at 450 nm and 690 nm. Results were calculated as a percentage of viable cells in the Puerariae radix-treated groups relative to the 0.5% DMSO-treated control.

4. Chemotaxis migration assay

Polyvinylpyrrolidone-free polycarbonate filters, pore size 12 m (Neuro Probe Inc. MD, USA), were coated with 0.1% gelatin and allowed to air dry. The lower compartments of Boyden chambers were filled with 1 106 cells in EBM plus 3 units/ml of heparin. The chambers were incubated at 37 C for 2 h, and then Puerariae radix was loaded into the upper compartments of the chambers being tested. The Boyden chambers were re-incubated at 37 C for 2 h, and then the filters were removed, fixed, and stained with Diff-Quik (Sysmex Co., Kobe, Japan). Cells on the lower surfaceof the filter were wiped off with a swab and the cells on the upper surface, which had migrated across the filter, were counted. Stained filters were photographed under a microscope (200, Axiovert 200, ZEISS, Germany), and the cells were quantified by counting the number of cells per field. Each assay was conducted in triplicate and experiments were repeated at least 3 times.

5. Tube formation assay on Matrigel

Unpolymerized Matrigel (Collaborative Biomedical Products, MA, USA) was added to 24-well plates, with a total volume of 300 $\mu\ell$ in each well, and allowed to polymerize for 30 min at 37 C. Various concentrations of Puerariae radix were plated onto the layer of Matrigel at a density of 1 105cells/well, in control medium. After 8 h, cells were photographed, and the extent of tube formation was analyzed using the NIH image program.

6. Measurement of bFGF

Cells were grown in 24-well plates until 90% confluent and then treated with 0.1, 1, 10 g/ml of Puerariae radix or the corresponding vehicle. After 72 h, culture supernatants were individually collected and frozen at 70 C before

immunoassay of bFGF with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., MN, USA). Samples were assayed in triplicate and calibrated against a bFGF standard (10640 pg/ml).

7. In vivo mouse Matrigel plug assay

Six-week-old male C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel alone, Matrigel plus 50 g/ml of Puerariae radix or 100 ng of bFGF per mouse, along with 10 units/ml of heparin. After 7 days, the mice were sacrificed, and the Matrigel plug was removed, fixed with 10% formalin and embedded in paraffin. Sections from the plugs were stained withhematoxylin and eosin for microscopic observation. Pathologists with no prior knowledge of the test agents examined the stained sections. To quantify the formation of new blood vessels, the amount of hemoglobin (Hb) present was measured using a hemoglobin reagent kit (Youngdong Diagnotics, Youngin, Korea) according to the supplier's protocol. The concentration of Hb was calculated by reference to a known amount of Hb provided in the kit.

8. Statistical analysis

The results are expressed as means SD, as calculated from the specified number of determinations. Data comparisons were performed using Student's t-test. Significance was defined as a pvalue of < 0.05.

Ⅲ. Results

1. Effects of Puerariae radix on proliferation

To investigate the angiogenic activity of Puerariae radix in detail, the dose of Puerariae radix with the optimum effect on endothelial cell growth was first determined. A range of 0100 g/ml Puerariae radix was applied to the HUVECs. Puerariae radix induced the growth of HUVECs in a dose-dependent manner, and endothelial cell proliferation was seen even at relatively low doses (Fig. 1). Puerariae radix at 0.01 g/ml significantly increased cell proliferation by 21.6%, and at 100 g/ml the proliferative effect was further increased to 45.5% (Fig. 1).

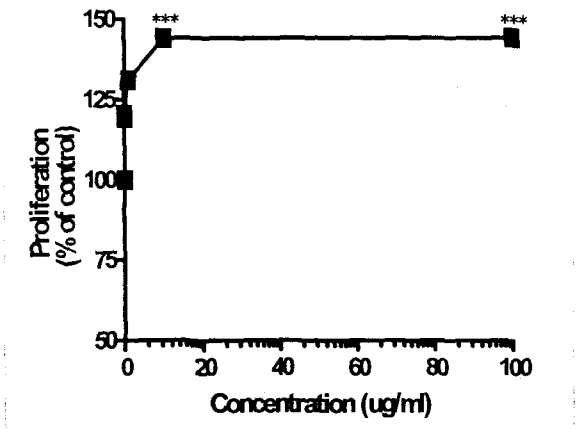

Fig. 1. Effects of Puerariae radix on proliferation of HUVECs. HUVECs were plated in 96-well plates, allowed to attach for 24 h, and then treated with different concentrations of Puerariae radix for 72 h. Cell proliferation was determined by a colorimetric BrdU assay. Data are expressed as percentage change of raw data. Results are shown as the mean ±SD of three experiments.
***P < 0.001 compared with control.

2. Effects of Puerariae radix on migration

The effects of Puerariae radix on endothelial cell migration were examined. Under Puerariae radix-free conditions, the number of migrating cells was 47.8 1.3 cells/field. Puerariae radix significantly induced cell migration in a dose-dependent manner, and at 25 g/ml, the number of migrating cells was 137.2 3.9 cells/field, a significant 2.87 fold induction compared with control (Fig. 2). In the positive control, bFGF at 100 ng/ml, the number of migrating cells was 179.6 3.4 cells/field, showing a 3.76-fold induction compared with control (Fig. 2).

3. Effects of Puerariae radix on tube-like formation

The inhibitory potency of Puerariae radix on the differentiation of endothelial cells into tube-like structures was tested. Under Puerariae radix-free condition, the number of HUVEC tube-like structures formed was 5.25 1.5 tubes/field; whereas in the presence of Puerariae radix at 10 g/ml, the number of tube-like structures formed was 27.7 5.3, a 5.3-fold stimulation compared with control. bFGF induced a 5.7-fold increase in the formation of HUVEC tube-like structures (Fig. 3).

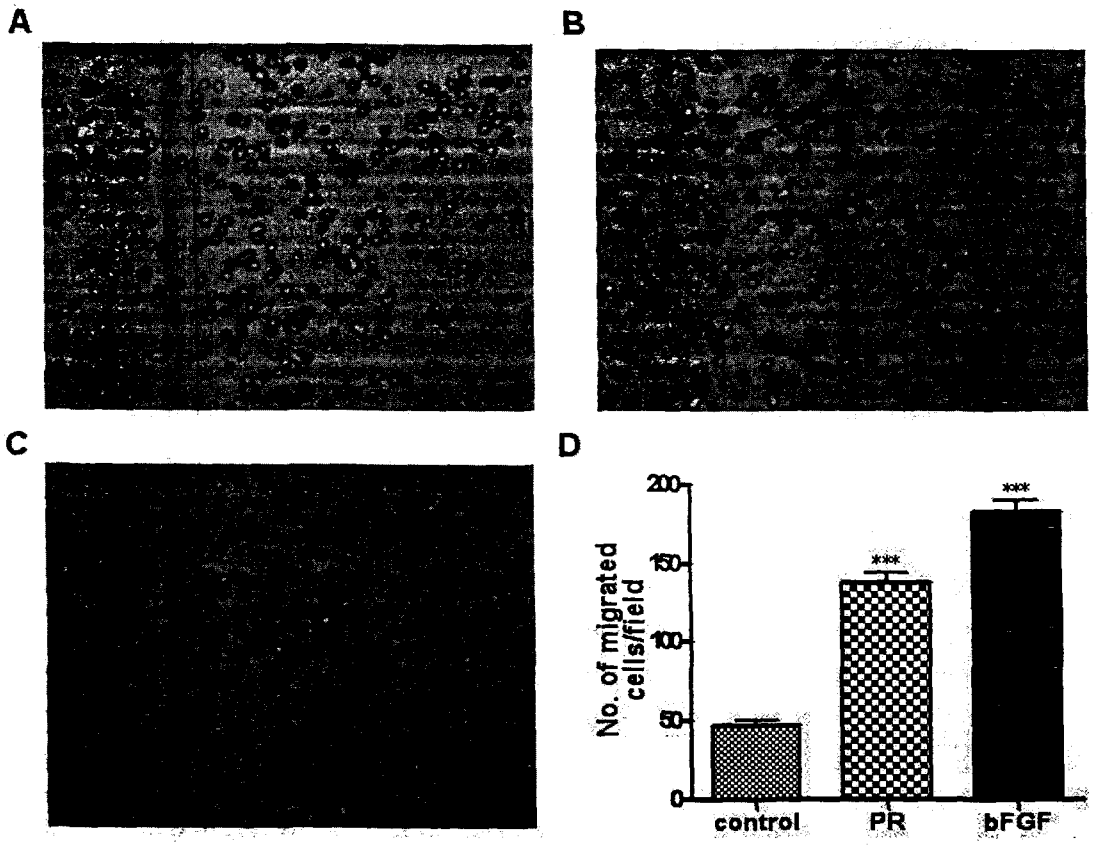

Fig. 2. Effects of Puerariae radix on migration of HUVECs. A modified Boyden chamber was used to assess migratory activity of untreated A; HUVECs (control), B; HUVECs exposed to 25 g/ml Puerariae radix (PR), C; 10 ng/ml bFGF (bFGF). Photomicrographs (magnification, 200) of a representative experimental result are shown. D; Migrated cells were counted in at least four fields after each assay, and results were expressed as the number of cells that migrated per field ± SD from three independent experiments.
***P < 0.001 compared with control.

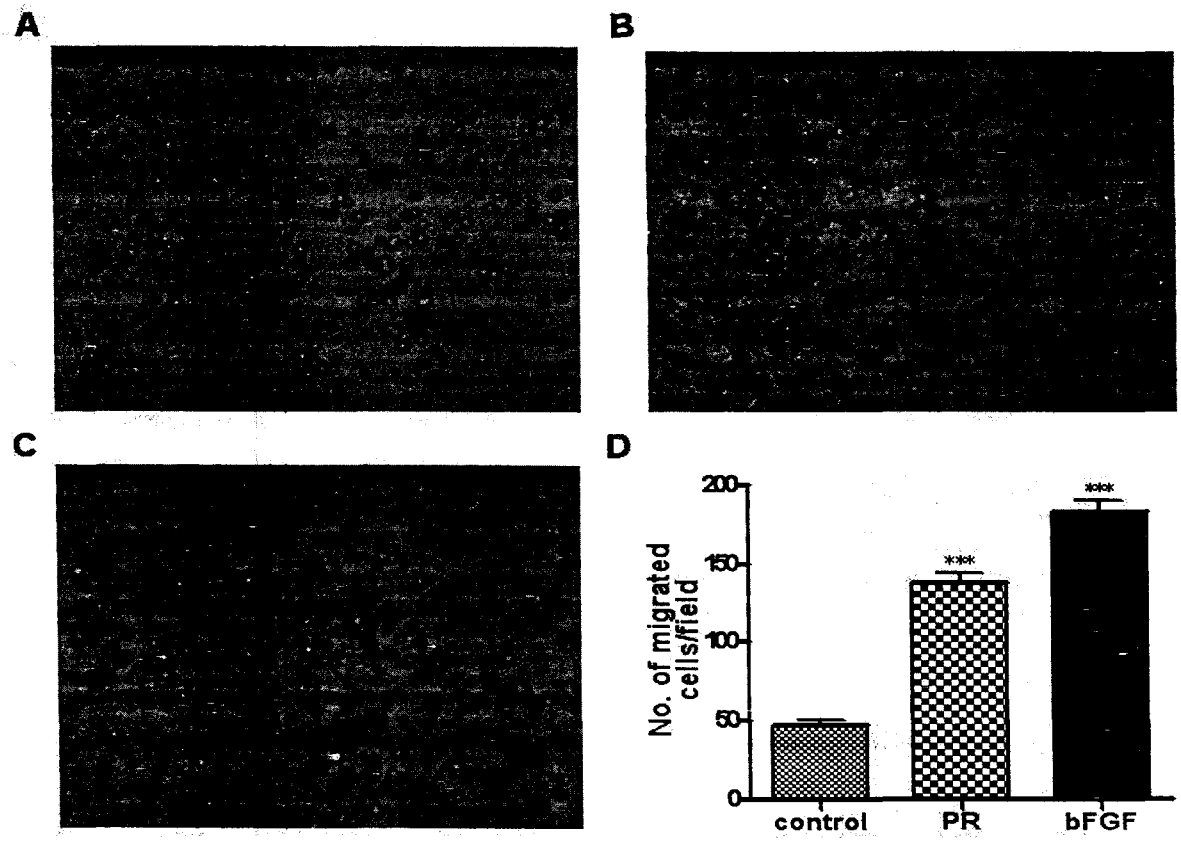

Fig. 3. Effects of Puerariae radix on tube-like formation in HUVECs. HUVECs were treated with different concentration of Puerariae radix on Matrigel. After incubation for 8 h and fixation, cells were observed under the microscope (magnification, 100) and photographed. A; Untreated HUVECs (control), B; HUVECs exposed to 0.1 g/ml Puerariae radix (PR), C; 10 ng/ml bFGF (bFGF) are shown. D; Tubes were counted per field in at least four fields after each experiment, and results were expressed as the number of tubes formed. Shown are the mean ±SD of three independent experiments.
***P < 0.0001 compared with control.

4. Effects of Puerariae radix on bFGF expression

To analyze further the effect of Puerariae radix on HUVEC proliferation, we tested the ability of Puerariae radix to modulate the production of bFGF. Puerariae radix dose-dependently increased bFGF expression significantly, by 1.8-fold at 0.01 g/ml, and by 4.23-fold at 10 g/ml (Fig. 4).

5. Effects of Puerariae radix on in vivo angiogenic activity

The angiogenic activity of Puerariae radix was investigated in an established in vivoangiogenesis model, the Matrigel plug assay. Matrigel plugs wereevaluated for tube/network formation and hemoglobin (Hb) content. In the histological examination, Matrigel control plugs containing bFGF showed tube/network formation. In addition, Puerariae radix strongly induced angiogenesis (Fig. 5A). The Hb content was 5.7 0.44 g/dl in control plugs and 11.6 3.4 g/dl in plugs containing bFGF at 100 ng. The Hb level in plugs containing Puerariae radix at 50 g/ml?? was significantly increased to 9.7 2.7 g/dl (Fig. 5B).

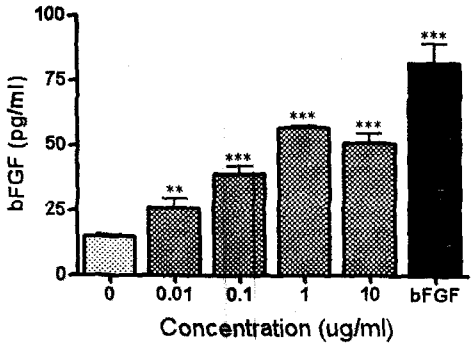

Fig. 4. Effects of *Puerariae radix* on production of bFGF by HUVECs. HUVECs were treated for 72 h with Puerariae radix at the concentrations indicated. bFGF in conditioned medium was assayed by ELISA. Values were determined in triplicate and calibrated against a bFGF standard. Each value represents mean ±SD.
** P < 0.01 and ***p < 0.001 compared with control.

Ⅳ. Discussion

Neovascularization is a complex process characterized by a cascade of events including activation and migration of endothelial cells, degradation and remodeling of basement membrane and surrounding extracellular matrix, endothelial cell proliferation, and neovessel formation[4,18-19]. The process is activated by the synthesis and release of angiogenic factors and/or the switching off of antiangiogenic factors by cells[18-19].

Despite a long history in oriental medicine of using Puerariae radix as a therapeutic agent in wound healing, ischemia, and the experience-based perception that such

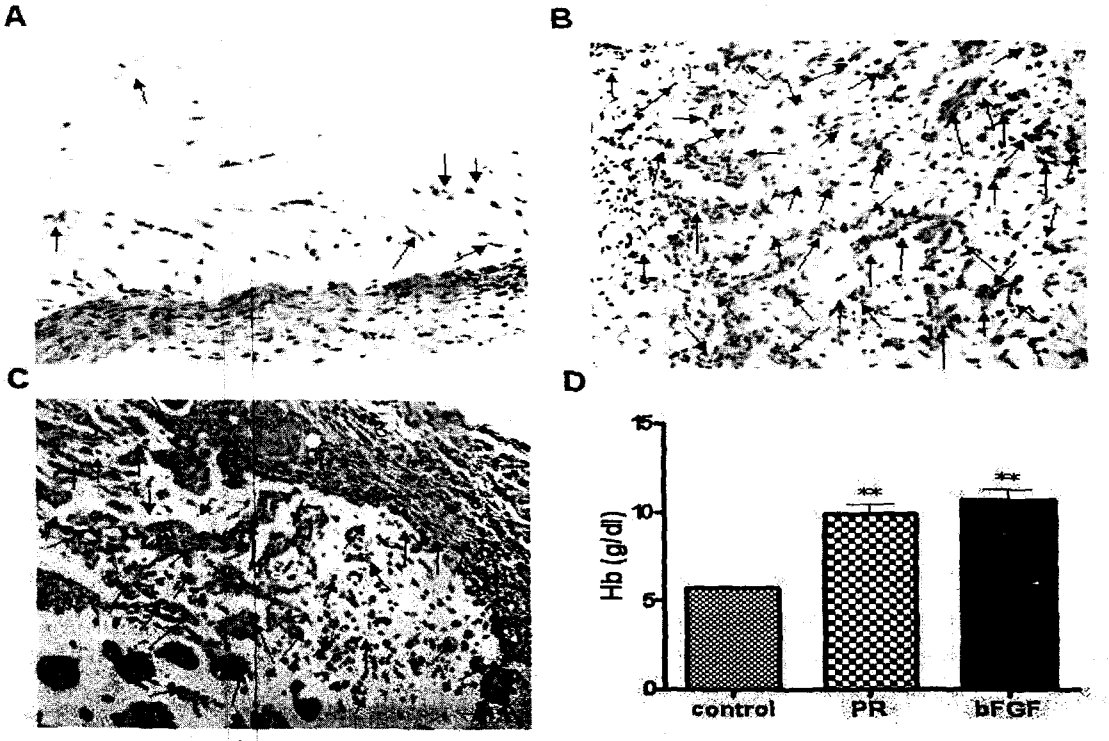

Fig. 5. Effects of Puerariae radix on angiogenesis Matrigel plug assay. Puerariae radix 50 g/ml or bFGF 100 ng plus 10 units/ml of heparin were mixed with Matrigel, and 0.5 ml of the mixture was injected subcutaneously into C57BL/6J mice. After 7 days, mice were sacrificed and the Matrigel plugs were excised. Sectioned Matrigel was stained with hematoxylin and eosin for microscopic observation. A; Control, B; Puerariae radix (PR), C; bFGF. Matrigel containing heparin alone was used as the negative control, and Matrigel mixed with bFGF and heparin was used as the positive control. D; Matrigel plugs were tested for hemoglobin (Hb) content to quantify the formation of functional blood vessels. Values shown are mean ±SD.
**p<0.01 compared with control.

treatments might be beneficial, there is no clear experimental evidence supporting this speculation. Therefore, we evaluated whether Puerariae radix would promote angiogenesis in vitro and in vivo.

In this study, we have shown that Puerariae radix moderately stimulates HUVEC proliferation (Fig. 1). To determine the specific effect of Puerariae radix on endothelial cells, we attempted chemotactic migration and capillary tube-like formation assays, and Puerariae radix strongly induced endothelial cell migration (Fig. 2) and tube-like formation (Fig. 3). Our data showed that the stimulation of HUVEC growth by Puerariae radix occurred at concentrations lower than the concentrations needed to induce cell migration and tube formation. It was thus expected that the induction of angiogenesis by Puerariae radix might initially be induced by stimulation of HUVEC proliferation.

To confirm the angiogenic activity of Puerariae radix through stimulation of HUVEC proliferation, we measured growth factors such as VEGF, bFGF and BMP-2. Puerariae radix slightly induced VEGF and BMP-2 expression (data not shown),and markedly increased bFGF in a dose-dependent manner (Fig. 4). This result implies that the expression of bFGF induced by Puerariae radix is closely related to the angiogenic activity of HUVECs. Recently, it has been reported that endogenous and exogenous FGF-2 accelerates wound healing in a chick embryo chorioallantoic membrane in vivo model[20-21]. In addition, it has been demonstrated that the healing of excisional skin wounds is delayed in mice lacking FGF-2[22]. Research in animal models of ischemiahas shown that administration of angiogenic growth factors promotes the development of neovascularization in collateral blood vessels[23-24]. Ex vivo gene therapy has enabled researchers to develop therapeutic angiogenesis strategies applied to an animal model of myocardial ischemia associated with capillary neovascularization[25]. In addition, in vivo models of bone fracture have demonstrated that the addition of exogenous FGF-2 to a fracture site or bone defect during the early healing stage accelerates fracture repair and bone formation[26-27].

We observed that Puerariae radix promotes in vivo angiogenesis in a model in which Puerariae radix-impregnated Matrigel implants led to an increase in tube/network formation and Hb content (Fig. 5). This in vivo result is supported by in vitro studies showing that Puerariae radix stimulates in vitro HUVEC cell proliferation and migration as well as the formation of capillary-like structures that play an essential role in the angiogenesis process.

V. Conclusion

These data present the first pharmacological evidence that Puerariae radix significantly induces angiogenesis in vitro and in vivo. In addition, it is expected that this study will allow better understanding of the relationship between angiogenesis and wound healing, and ischemia control by Puerariae radix in oriental medicines. Taken together, these results show that Puerariae radix is a potent angiogenic agent and a promising drug for the induction of neovascularization.

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