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원 저

Effects of *Astragalus Membranaceus* on Angiogenesis

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국문초록

황기가 혈관 형성에 미치는 영향

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목적 : 황기가 혈관 신생 작용이 있는지에 관하여 관찰한다. 황기는 상처의 치유나 허혈성 질환에 효과를 나타내는 것으로 알려져 있다. 이러한 효과가 황기의 혈관 신생 작용과의 관련성을 이해하며 향후 임상에 쓰일 수 있는 황기 약침액 개발을 위한 기초 자료를 목표로 한다.

방법 : 황기의 혈관 신생 작용의 관찰을 위하여 human umbilical vein endothelial cells(HUVECs)와 Matrigel angiogenesis model을 이용하여 연구하였다.

결과 : 황기는 용량에 따라서 HUVECs의 증식을 나타내었다. 또한 혈관 내피 세포의 이동과 관형 형성을 보였다. 혈관 신생 물질인 basic fibroblast growth factor(bFGF)가 황기에 의해 증가하였다. Matrigel angiogenesis model에서 황기는 조직학적으로 혈관 형성을 촉진하였으며, 헤모글로빈의 증가를 나타내었다.

핵심단어 : *Astragalus membranaceus*, Angiogenesis, HUVECs, Matrigel, basic fibroblast growth factor

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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[1,2]. 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)[2,5,6]. Growth factors have 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[13].

Angiogenesis is important for successful fracture healing[14]. One of the most important angiogenic factors is the vascular endothelial growth factor(VEGF), a glycosylated protein of 4648 kDa composed of two disulfide-linked subunits30). Five different VEGF isoforms with 120, 144, 164, 188, and 205 amino acids can be generated as a result of alternative splicing from the single VEGF gene in rats. These isoforms differ in their molecular mass and in their biological properties such as their ability to bind to heparinsulfate proteoglycans and to different VEGF receptors(VEGFR)[15]. The signaling tyrosine kinase receptors VEGFR-1(flt-1) bind VEGF 120, VEGF164, and VEGFR-2 in addition to VEGF144[16,17].

The roots of *Astragalus membranaceus*(Huangqi) are among the most popular health-promoting herbs in oriental medicine. In traditional folk medicine, *Astragalus membranaceus* has been shown to have effects on general health, ischemic heart disease, urination, and as an antinephritis agent. Recently, it was reported that the polysaccharide fraction from *Astragalus membranaceus* enhanced immune function through activation of B cells and macrophages in mice[17]. In addition, it has been demonstrated that *Astragalus membranaceus*, alone and in combination, prevents bone loss in ovariectomized rats[18,19].

The aim of this study is to identify and characterize whether *Astragalus membranaceus* could induces angiogenesis in HUVECs and Matrigel plug assay.

Therefore, this study may allow better understanding of the relationship between angiogenesis and wound healing, and ischemia control by Astragalus membranaceus in oriental medicines and be the basement of developing herbal acupuncture for treatment of wound healing, ischemia and bone fracture.

Ⅱ. Materials and Methods

1. Preparation of Astragalus membranaceus extract

The root of *Astragalus membranaceus* was purchased from Oriental medicine of KyungHee University. Two thousand grams of *Astragalus membranaceus* was extracted with 70%(v/v) ethanol - water at 60°C for 24h. The extract was then filtered with 10uM cartridge paper, the ethanol was removed by vacuum rotary evaporation(Eyela, Japan). The concentrate was

freeze-dried and its yield was 12.1%(242g). 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 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 (Sigma - Aldrich Co., MO. USA) in PBS for 10 min at 37°C. After pelleting and resuspending the cells, they were plated in a 75cm^2 tissue culture flask coated with 0.1% gelatin, cultured with EGM TM-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 5×103cells/well in EGMTM-2 medium in 96-well plates. After 24h, the medium was removed and replaced with EBM(Cambrex Inc., MD, USA) plus 2% FBS, and 3units/ml of heparin(control medium). Cells were treated with 0.01, 0.1, 1, 10, 100μg/ml of *Astragalus membranaceus* 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 6h 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 450nm and 690nm. Results were calculated as a percentage of viable cells in the *Astragalus membranaceus*- treated groups relative to the 0.5% DMSO-treated control.

4. Chemotaxis migration assay

Polyvinylpyrrolidone-free polycarbonate filters, pore size 12μm(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 × 106cells in EBM plus 3 units/ml of heparin. The chambers were incubated at 37°C for 2h, and then *Astragalus membranaceus* was loaded into the upper compartments of the chambers being tested. The Boyden chambers were re-incubated at 37°C for 2h, and then the filters were removed, fixed, and stained with Diff-Quik(Sysmex Co., Kobe, Japan). Cells on the lower surface of 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 3times.

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 30min at 37°C. Various concentrations of *Astragalus membranaceus* were plated onto the layer of Matrigel at a density of 1 × 105cells/well, in control medium. After 8h, 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, 10μg/ml of *Astragalus membranaceus* or the corresponding vehicle. After 72h, 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(10 - 640pg/ml).

7. In vivo mouse Matrigel plug assay

Six-week-old male C57BL/6mice were injected subcutaneously with 0.5ml of Matrigel alone, Matrigel plus 50μg/ml of *Astragalus membranaceus* or 100ng of bFGF per mouse, along with 10 units/ml of heparin. After 7days, the mice were sacrificed, and the Matrigel plug was removed, fixed with 10% formalin and embedded in paraffin. Sections from the plugs were stained with hematoxylin 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 p value of < 0.05.

Ⅲ. RESULTS

1. Effects of *Astragalus membranaceus* on proliferation

Astragalus membranaceus induced the growth of HUVECs in a dose-dependent manner, and endothelial cell proliferation was seen even at relatively low doses(Fig. 1). *Astragalus membranaceus* at 0.01μg/ml significantly increased cell proliferation by 21.2%, and at 100 μg/ml the proliferative effect was further increased to 34.5%(Fig. 1).

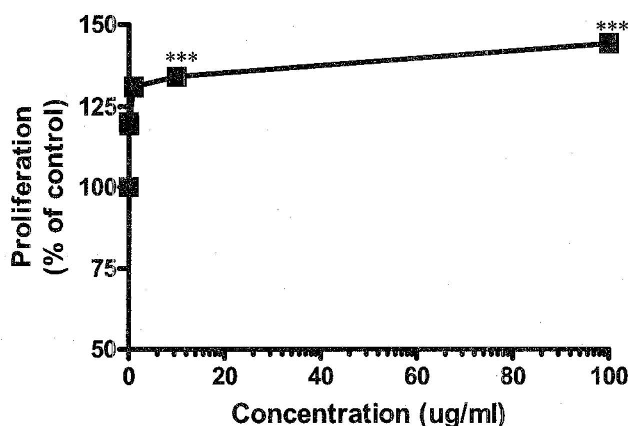

Fig. 1. Effects of *Astragalus membranaceus* on proliferation of HUVECs
HUVECs were plated in 96-well plates, allowed to attach for 24h, and then treated with different concentrations of *Astragalus membranaceus* for 72h. Cell proliferation was determined by a colorimetric BrdU assay. Data are expressed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

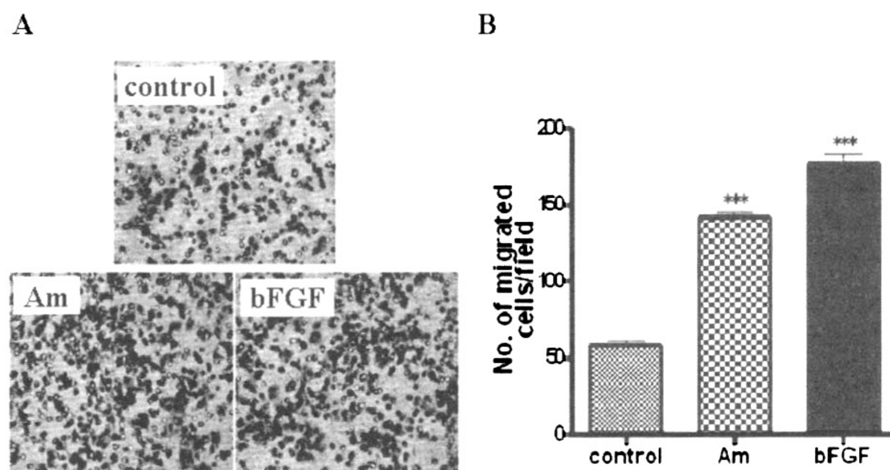

Fig. 2. Effects of *Astragalus membranaceus* on migration of HUVECs
(A) A modified Boyden chamber was used to assess migratory activity of untreated HUVECs(control), HUVECs exposed to 25μg/ml *Astragalus membranaceus*(Am), or 10ng/ml bFGF(bFGF). Photomicrographs(magnification, 200 ×) of a representative experimental result are shown. (B) Migrated cells were counted in at least four fields after each assay, and data are expressed as the number of cells per field. Results are shown as the mean ±SD of three independent experiments. ***P < 0.001 compared with control.

2. Effects of *Astragalus membranaceus* on endothelial cell migration

Under *Astragalus membranaceus*-free conditions, the number of migrating cells was 58.8 ± 2.3 cells/field. *Astragalus membranaceus* at 25μg/ml significantly induced cell migration, the number of migrating cells was 142 ± 3.5cells/field, a significant 2.4-fold induction compared with control(Fig. 2). In the positive control, bFGF at 100ng/ml, the number of migrating cells was 176.8 ± 4.5cells/field, showing a 3.0-fold induction compared with control (Fig. 2).

3. Effects of *Astragalus membranaceus* on tube-like formation

Under *Astragalus membranaceus*-free condition, the number of HUVEC tube-like structures formed was 5.25 ± 1.5tubes/field; whereas in the presence of *Astragalus membranaceus* at 10μg/ml, the number of tube-like structures formed was 30.5 ± 4.3tubes/field, a 5.8-fold stimulation compared with control. bFGF induced a 5.7-fold increase in the formation of HUVEC tube-like structures(Fig. 3).

4. Effects of *Astragalus membranaceus* on bFGF expression

Astragalus membranaceus dose-dependently increased bFGF expression significantly, by 21.2% at 0.01 μg/ml, and by 34.5% at 10μg/ml(Fig. 4).

5. Effects of *Astragalus membranaceus* on in vivo angiogenic activity

In the histological examination, Matrigel control plugs containing bFGF showed tube/network formation. In addition, *Astragalus membranaceus* strongly induced angiogenesis(Fig. 5A). The Hb content was 5.7 ± 0.44g/dl in control plugs and 9.8 ± 6.4g/dl in plugs containing bFGF at 100ng. The Hb level in plugs containing *Astragalus membranaceus* at 50μg/ml was significantly increased to 11.5 ± 3.1g/dl(Fig. 5B).

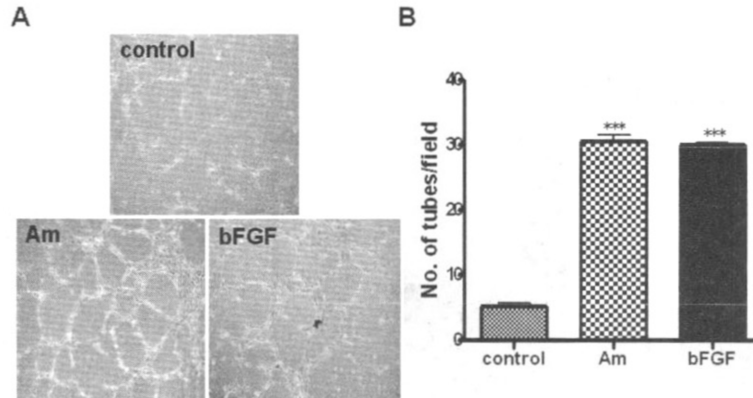

Fig. 3. Effects of *Astragalus membranaceus* on tube-like formation in HUVECs

(A) HUVECs were treated with different concentration of *Astragalus membranaceuson* Matrigel. After incubation for 8h and fixation, cells were observed under the microscope (magnification, 100 ×) and photographed. Untreated HUVECs(control), HUVECs exposed to 0.1μg/ml *Astragalus membranaceus* (Am), or 10ng/ml bFGF (bFGF) are shown. (B) Tubes were counted per field in at least four fields after each experiment, and results were expressed as the number of tubes formed. Results are shown as the mean ±SD of three independent experiments. ***P < 0.001 compared with control.

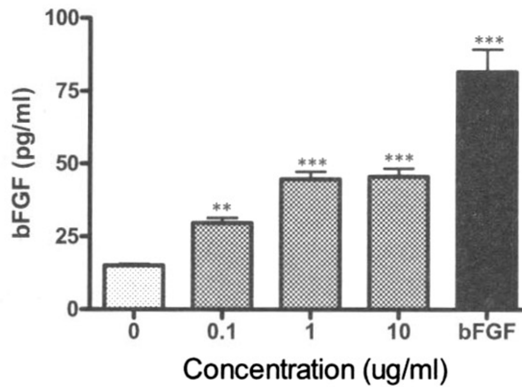

Fig. 4. Effects of *Astragalus membranaceus* on production of bFGF by HUVECs

HUVECs were treated for 72h with *Astragalus membranaceus* 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.

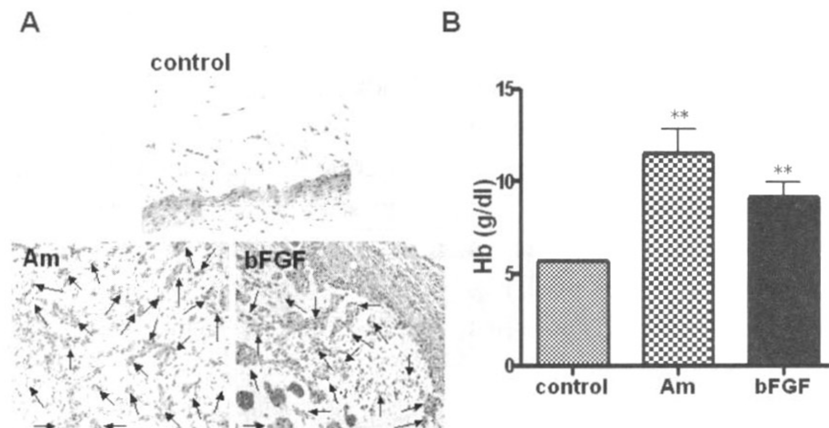

Fig. 5. Effects of *Astragalus membranaceus* on angiogenesis Matrigel plug assay
Astragalus membranaceus 50μg/ml or bFGF 100ng plus 10units/ml of heparin were mixed with Matrigel, and 0.5 ml of the mixture was injected subcutaneously into C57BL/6J mice. After 7days, mice were sacrificed and the Matrigel plugs were excised. Sectioned Matrigel was stained with hematoxylin and eosin for microscopic observation. (A) Control, *Astragalus membranaceus*(Am), and bFGF. Matrigel containing heparin alone was used as the negative control, and Matrigel mixed with bFGF and heparin was used as the positive control. (B) 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.

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[2,20,21]. These cascades are activated by the release of angiogenic factors and the switching off of antiangiogenic factors by cells[21]. 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)[2,5,6].

Growth factor 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]

In Traditional Chinese Medicine(TCM), *Astragalus membranaceus* is classified as an herb that tonifies the qi and is indicated for symptoms of spleen qi deficiency such as diarrhea, fatigue, and lack of appetite. It also raises the yang qi of the spleen and stomach, thus addressing prolapses of organs such as the uterus, stomach, or anus. In this capacity it can also address uterine bleeding. *Astragalus membranaceus* tonifies the lung qi and is used in cases of frequent colds, spontaneous sweating, and shortness of breath. Other traditional indications include wasting disorders, night sweats, chronic ulcerations and sores,1 numbness and paralysis of the limbs, and edema. Its properties are sweet and slightly warm[22].

Current Western applications of *Astragalus*

membranaceus are primarily for restoring and strengthening the immune response, enhancing cardiovascular function, and increasing vitality. Indications supported by clinical trials include impaired immunity, adjunctive cancer treatment, and viral infections, including the common cold and cervical erosion associated with Herpes simplex. Western preparations include dried root for decoction, liquid extract, tablets, and powdered root[23].

Despite a long history in oriental medicine of using *Astragalus membranaceus* as a therapeutic agent in wound healing, ischemia, and the experience-based perception that such treatments might be beneficial, there is no clear experimental evidence supporting this speculation. Therefore, this study evaluated whether *Astragalus membranaceus* would promote angiogenesis in vitro and in vivo.

This study has shown that *Astragalus membranaceus* moderately stimulates HUVEC proliferation. To determine the specific effect of *Astragalus membranaceus* on endothelial cells, this study attempted chemotactic migration and capillary tube-like formation assays, and *Astragalus membranaceus* strongly induced endothelial cell migration and tube-like formation. This data showed that the stimulation of HUVEC growth by *Astragalus membranaceus* 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 *Astragalus membranaceus* might initially be induced by stimulation of HUVEC proliferation.

To confirm the angiogenic activity of *Astragalus membranaceus* through stimulation of HUVEC proliferation, growth factors was measured such as VEGF, bFGF and BMP-2. *Astragalus membranaceus* slightly induced VEGF and BMP-2 expression, and markedly increased bFGF in a dose-dependent manner. This result implies that the expression of bFGF induced by *Astragalus membranaceus* 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[15]. In addition, it has been demonstrated that the healing of excisional skin wounds is delayed in mice lacking FGF-2[24]. Research in animal models of ischemia has shown that administration of angiogenic growth factors promotes the development of neovascularization in collateral blood vessels[25,26]. Ex vivo gene therapy has enabled researchers to develop therapeutic angiogenesis strategies applied to an animal model of myocardial ischemia associated with capillary neovascularization[27].

Astragalus membranaceus promoted in vivo angiogenesis in a model in which *Astragalus membranaceus*-impregnated Matrigel implants led to an greatly increase in tube/network formation and Hb content compared with bFGF. This in vivo result is supported by in vitro studies showing that *Astragalus membranaceus* 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.

These data present the first pharmacological evidence that *Astragalus membranaceus* 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 *Astragalus membranaceus* in oriental medicines. Taken together, these results show that *Astragalus membranaceus* is a potent angiogenic agent and a promising drug for the induction of neovascularization.

Fracture repair is a complex physiological process, which can be enhanced through therapeutic approaches. The fracture repair process is divided into distinguishable stages: the initial hematoma, angiogenesis and chondrogenesis, endochondral bone formation, and bone remodeling. At the initial phase, inflammatory cells, macrophages, and degranulating platelets infiltrate into the fracture site. Various growth factors and cytokines released from these cells play key roles as initiators of the

fracture repair process resulting in osteoprogenitor cell differentiation and proliferation, and angiogenesis[28-30].

Angiogenesis is a tightly regulated process in which the actions of proangiogenic factors are counterbalanced by the actions of anti-angiogenic factors. During bone development or regeneration, as in many other tissues, VEGF-A and their receptors appear to be the major proangiogenic factor required to induce new vessel growth52). The initiation of formation of new blood vessels mediated by VEGF and their receptors is essential for fracture healing[28-32].

Previously reported, in addition to its role in bone angiogenesis, VEGF-A stimulates both diffrentiation and migration, but not proliferation in osteoblasts[33,34]. VEGF-A affects bone resorption by direct effects on osteoclast survival and by increasing the number of osteoclasts during neovascularization[35]. Increased osteoclast resorption is also associated with several severe bone diseases including osteoporosis, intervertebral herniated discs, inflammatory-related rheumatoid arthritis, bone cancers, periodontal, and Paget's disease[35,36].

Recently, it was reported that the polysaccharide fraction from *Astragalus membranaceus* enhanced immune function through activation of B cells and macrophages in mice[33]. In addition, it has been demonstrated that *Astragalus membranaceus*, alone and in combination, prevents bone loss in ovariectomized rats[34].

Therefore, this study may be the basement of developing herbal acupuncture for treatment of wound healing and ischemic cardiovascular disease. Also Further study for effects of *Astragalus membranaceus* on osteogenesis may be necessary.

V. Conclusions

Astragalus membranaceus significantly increased human umbilical vein endothelial cells(HUVECs) proliferation in a dose-dependent manner. In addition, *Astragalus membranaceus* 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 *Astragalus membranaceus*. The angiogenic activity of *Astragalus membranaceus* was confirmed using an in vivo Matrigel angiogenesis model, showing promotion of blood vessel formation. These results suggest that *Astragalus membranaceus* is a potent angiogenic agent, and a promising drug, for the induction of neovascularization.

Astragalus membranaceus plays the important role on angiogenesis in tissue regeneration through enhanced by growth factors(bFGF, VEGF) and possibly lead to the development of tissue-regeneration drug.

Ⅵ. References

1. Findlay JK. Angiogenesis in reproductive tissues. Journal of Endocrinology 111. 1986 : 357-66.
2. Folkman J, Shing Y. Angiogenesis. Journal of Biological Chemistry. 1992 ; 10931-4.
3. Ausprunk DH, Folkman J. Migration and proliferation of endothelial cells in performed and newly formed vessels during tumor angiogenesis. Microvascular Research. 1977 ; 53-65.
4. Furcht LT. Critical factors in controlling angiogenesis : cell products, cell matrix, and growth factors. Laboratory Investigation. 1986 ; 505-9.
5. Ferrara N, Houck K, Jakeman L, Leung, DW. Moecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrine Reviews. 1992 ; 18-32.
6. Burgess WH, Maciag T. The heparin-

binding (fibroblast) growth factor family of proteins. Annual Review of Biochemistry. 1989 : 575-602.

7. Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D. Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature ; 1987 ; 257-9.
8. Voldavsky I, Folkman J, Sullivan R, Fridman R, Ishai-michaeli R, Sasse J, Klagsbrun M. Endothelial cell-derived basic fibroblast growth factor : Synthesis and deposition into subendothelial extracellular matrix. Proceedings National Academic Science USA. 1987 ; 2292-6.
9. Tsuboi R, Rifkin DB. Recombinant basic fibroblast growth factor stimulates wound healing in healing-impared db/db mice. The Journal of Experimental Medicine. 1990 ; 245-51.
10. Murayama T, Tepper OM, Silver M, Ma H, Losordo DW, Isner JM, Asahara T, Kalka C. Determination of bone marrow- derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. Experimental Hematology. 2002 ; 967-2.
11. Horvath KA, Doukas J, Lu CY, Belkind N, Greene R, Pierce GF, Fullerton DA. Myocardial functional recovery after fibroblast growth factor 2 gene therapy as assessed by echocardiography and magnetic resonance imaging. The Annals of Thoracic Surgery. 2002 ; 481-6.
12. Nakamura T, Hara Y, Tawaga M, Tamura M, Yuge T, Fukuda H, Nigi H. Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. Journal of Bone and Mineral Research. 1998 ; 942-9.
13. Silvestre JS, Levy BI. The renin-angiotensin system and post-ischemic angiogenesis. Bulletin of Academic National Medicine. 2004 ; 649-59.
14. Frost HM. The biology of fracture healing. An overview for clinicians, part I. Clin Orthop. 1989 ; 283-93.
15. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. J Mol Med. 1999 ; 527-43.
16. Fong DH, Rossant J, Gersenstein M, Breitman ML. Role of the Flt-1receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995 ; 376-7.
17. Neufeld G, Cohen T, Gengrinovitch S, Poltrak Z. Vascular endothelial growth factor and its receptors. FASEB J. 1999 ; 922.
18. Shao BM, Xu W, Dai H, Tu P, Li Z, Gao XM. A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a chinese medicinal herb. Biochemical and Biophysical Research Communications. 2004 ; 1103- 11.
19. Kim C, Ha H, Lee JH, Kim JS, Song K, Park SW. Herbal extract prevents bone loss in ovariectomized rats. Archives of Pharmacology and Research. 2003 ; 917-24.
20. D'Amore PA, Thompson RN. Mechanism of angiogenesis. Annual Review of Physiology. 1987 ; 453-464.
21. Folkman J, Klagsburn, M. Angiogenic factors. Science. 1987 ; 442-7.
22. Bensky D, Gamble A. Chinese Herbal Medicine : Materia Medica, Revised Edition. Seattle, WA : Eastland Press. 1993.
23. Mills S, Bone K. Principles and Practice of Phytotherapy. Edinburgh, Scotland : Churchill Livingstone. 2000 ; 273-279.
24. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proceedings National Academic Science USA. 1998 ; 5672-7.
25. Laham RJ, Garcia L, Baim DS, Post M, Simons M. Therapeutic Angiogenesis using basic fibroblast growth factor and vascular

endothelial growth factor using various delivery strategies. Current International Cardiology Report 1. 1999 ; 228-33.
26. Marui A, Kanematsu A, Yamahara K, Doi K, Kushibiki T, Yamamoto M, Itoh H, Ikeda T, Tabata Y, Komeda M. Simultaneous application of basic fibroblast growth factor and hepatocyte growth factor to enhance the blood vessels formation. Journal of Vascular Surgery 41. 2005 ; 82-90.
27. Ninomiya M, Koyama H, Miyata T, Hamada H, Miyatake S, Shigematsu H, Takamoto S. Ex vivo gene transfer of basic fibroblast growth factor improves cardiac function and blood flow in a swine chronic myocardial ischemia model. Gene Therapy. 2003 ; 1152-60.
28. Brookes M, Revell WJ. Blood supply of bone. Springer. London. 1998.
29. Gerber HP, Vu TH, Ryan AM, Kolwalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. 1999 : 623-8.
30. Tatsuyama K, Maezawa Y, Baba H, Imamura Y, Fukuda M. Expression of various growth factors for cell proliferation and cyto differentiation during fracture repair of bone. Eur J Histochem. 2000 ; 269-78.
31. Bolander ME. Regulation of fracture repair by growth factors. Proceedings of the Society for Experimental Biology & Medicine. 1992 ; 165-70.
32. H.-P. Gerber, N. Ferrara, Angiogenesis and bone growth, Trends Cardiovasc. Med. 2000 ; 223-8.
33. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kiekens L, Gerstenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declerq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996 ; 435-9.
34. Fong DH, Rossant J, Gersenstein M, Breitman ML. Role of the Flt-1receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995 ; 66-70.
35. Shalaby F, Rossant J, Yamaguchi TP, Gerstenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995 ; 62-6.
36. Gerber HP, Vu TH, Ryan AM, Kolwalski J, Werb Z, Ferrara N VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. 1999 ; 623-8.