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Stability of angiogenic agents, ginsenoside Rg_1 and Re, isolated from *Panax ginseng*: *In vitro* and *in vivo* studies

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Abstract

The study was designed to investigate the stability of ginsenoside Rg_1 (Rg_1) and Re (Re), two natural herbal compounds isolated from *Panax ginseng*, based on their activity to promote angiogenesis *in vitro* and *in vivo*. After being treated at different temperatures, pHs, and solvent species for distinct durations, the remaining activities of Rg_1 and Re on human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation were examined *in vitro*. Additionally, the remaining activity of each treated test agent, mixed in a growth factor-reduced Matrigel, in stimulating angiogenesis was evaluated subcutaneously in a mouse model. Basic fibroblast growth factor (bFGF) was used as a control. It was found *in vitro* that HUVEC proliferation, migration in a Transwell plate, and tube formation on Matrigel were all significantly enhanced in the presence of bFGF, Rg_1, or Re. However, after being treated at different temperatures, pHs, or solvent species, the remaining activity of bFGF on HUVEC behaviors reduced significantly. This observation was more significant with increasing the duration of treatment. In contrast, the activities of Rg_1 and Re remained unchanged throughout the entire course of the study. The *in vivo* results observed on day 7 after implantation showed that the blank control (Matrigel alone) was slightly vascularized. In contrast, the density of neo-vessels in the Matrigel plug mixed with bFGF, Rg_1, or Re was significantly enhanced. However, after being treated, the density of neo-vessels was significantly reduced in the Matrigel plug mixed with bFGF, while those of Rg_1 and Re remained unchanged. The aforementioned results suggested that Rg_1 and Re could be a novel group of nonpeptide angiogenic agents with a superior stability and may be used for the management of tissue regeneration.
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Keywords: Ginsenoside Rg_1; Ginsenoside Re; bFGF; Angiogenic agent; Stability

1. Introduction

Vascularization in extracellular matrices (ECMs) to support the metabolic needs of the engineered tissues is generally a prerequisite for achieving appropriate tissue regeneration and function (Kelm et al., 2004; Pieper et al., 2002). Investigations have incorporated angiogenic factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), or platelet derived growth factor (PDGF) to stimulate angiogenesis in ECMs (Ley et al., 2004). However, the biological activity of these protein-type growth factors may not last long due to their poor stability (Prisell et al., 1992; Shea et al., 1999; Wang, 2005), resulting from denaturation and deactivation of proteins during the formulation process with an ECM or less than optimal storage conditions (Tabata, 2003).

Panax ginseng has long been used in herbal medicine in the repair of intractable skin ulcers of patients with diabetes mellitus (Morisaki et al., 1995). Angiogenesis is known to play an important role in the repair of ulcers. Ginsenoside Rg_1 (Rg_1) and Re (Re) are two of the active components of saponin in *Panax ginseng* (Lee et al., 1997; Scott et al., 2001).

Rg_1 has an estrogen-like activity and may be classified as a novel class of potent phytoestrogen (Chan et al., 2002). It is known that estrogen directly modulates angiogenesis via effects on endothelial cells (Morales et al., 1995). Experimentally, Re was shown to stimulate the activity of nitric oxide synthase (NOS) significantly (Scott et al., 2001). It was reported that nitric oxide (NO) is a downstream mediator in the angiogenic response to a variety of growth factors, but the mechanism by which

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NO promotes neo-vessel formation is still not clear (Babaei and Stewart, 2002). Sengupta et al. (2004) suggested that in addition to promoting the synthesis of NOS enzyme, Rg_1 can activate through the phosphatidylinositol-3 kinase → phospho-Akt → NOS pathway.

As aforementioned, the biological activity of angiogenic agents may get denatured and deactivated throughout their loading process in an ECM or during storage (Tabata, 2003). Therefore, effects of temperature, pH, and solvent species (the parameters used to load angiogenic agents in an ECM or during storage, Fransson et al., 1997; Zour et al., 1992) on the stability of Rg_1 and Re must be investigated, based on their activity to promote angiogenesis *in vitro* and *in vivo*. In the study, the remaining activities of Rg_1 and Re after being treated at different temperatures, pHs, or solvent species for distinct durations on human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation were examined *in vitro*. Additionally, the remaining activity of each treated test agent, mixed in a growth factor-reduced Matrigel, in stimulating angiogenesis was evaluated subcutaneously in a mouse model. bFGF, a commonly used angiogenic agent in tissue engineering, was used as a control.

2. Materials and methods

2.1. Materials

bFGF (with heparin binding), Rg_1, and Re (Fig. 1) were purchased from PeproTech Inc. (Rocky Hill, NJ, USA), Nacalai Tesque Inc. (Kyoto, Japan), and Extrasynthese Co. Ltd. (Zone Industrielle, Lyon Nord, France), respectively. Matrigel obtained from Becton Dickinson Biosciences (growth factor-reduced, San Jose, CA, USA) was used for all the related experiments. All other chemicals and reagents used were of analytical grade. Stock solutions of bFGF (500 ng/ml), Rg_1 (1.5 mg/ml), and Re (1.5 mg/ml) were prepared separately in 20% aqueous ethanol and used for all experiments. It should be noted that the final content of ethanol in the growth medium was less than 0.5% (v/v), which would not interfere with the test system (Zhang et al., 2001). Furthermore, all the control groups contained the same amount of ethanol as those in the compound-treated groups.

2.2. Treatments of test agents

In the study, bFGF, Rg_1, and Re were treated and kept separately at different temperatures, pHs, or solvent species for distinct periods. For the temperature treatment, the prepared stock solutions of bFGF, Rg_1, and Re were kept separately at 4, 25, 37, and 50 °C for 3, 7, and 14 days. For the pH treatment, each stock solution was buffered at pH 4.0 (sodium acetate–acetic acid buffer), pH 7.4 (phosphate buffered saline, PBS), or pH 10.0 (carbonate buffer) at 37 °C for 3 and 7 days. For the solvent treatment, bFGF, Rg_1, and Re were prepared in 20% aqueous ethanol, anhydrous ethanol, and dimethyl sulfoxide (DMSO) separately at 37 °C for 3 and 7 days. Ethanol and DMSO have been frequently used as solvents to dissolve experimental drugs and compounds that do not easily dissolve in water or saline (Castro et al., 1995). In the study it was found that Re is rather hydrophobic and its solubility in water is limited. Subsequently, the remaining activity of each treated test agent on HUVEC proliferation, migration, and tube formation was studied *in vitro*.

2.3. In vitro study

HUVECs (Cascade Biologies, Portland, OR, USA) were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in Medium 200 supplemented with low serum growth supplement (LSGS, Cascade Biologies). All experiments were carried out with the same batch of HUVECs (passages 4–6). Confluent HUVEC monolayers were used in the proliferation, migration, and tube formation assays as described below. The concentrations of test agents used in the *in vitro* study were: 10 ng/ml for bFGF and 30 μg/ml for Rg_1 and Re. The former concentration was commonly used in the literature to evaluate the angiogenic activity of bFGF *in vitro* (Nie et al., 2000). Additionally, it was reported that effects of Re on HUVEC

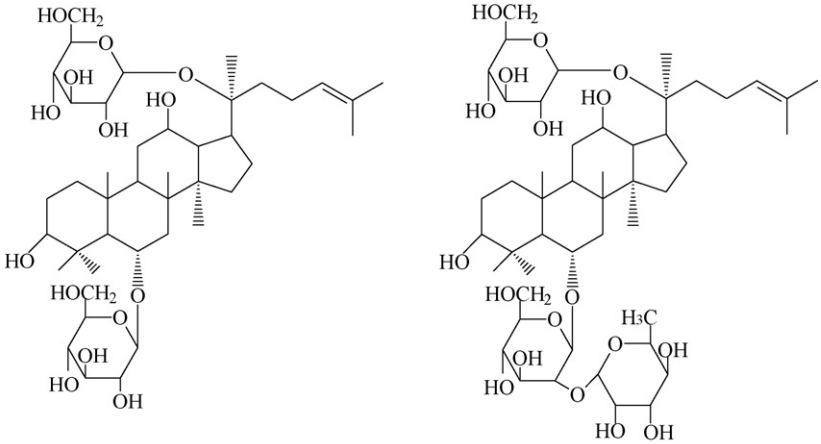

Fig. 1. Chemical structures of ginsenoside Rg_1 and Re.

behaviors were dose-dependent and reached a maximal level at a concentration of about 30 μg/ml (Huang et al., 2005). Therefore, the concentration used for Rg_1 and Re was 30 μg/ml.

Effects of each test agent on HUVEC proliferation, migration, and tube formation were investigated *in vitro*. Details of methodologies used in the study were similar to those reported in the literature and described in our previous studies (Huang et al., 2005; Liang et al., 2005; Morales et al., 1995).

2.4. In vivo angiogenesis assessment by the Matrigel plug assay

The remaining activity of each treated test agent (Hall et al., 2001) was also investigated *in vivo*. The study was conducted under aseptic conditions using a mouse model (6-week-old male C57/BL6 mice). Animal care and use was performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. In total, 35 mice were used and separated into seven groups ($n = 5$ for each studied group): Matrigel alone (MG/Control); those mixed with fresh bFGF (before being treated, 0.5 μg/ml, MG/bFGF), Rg_1 (50 μg/ml, MG/Rg_1), and Re (50 μg/ml, MG/Re); and those mixed with bFGF (0.5 μg/ml, MG/bFGF-Day 7), Rg_1 (50 μg/ml, MG/Rg_1-Day 7), and Re (50 μg/ml, MG/Re-Day 7) after being treated at 37 °C for 7 days. The concentration applied for each test agent *in vivo* was based on those used in the literature (Klement et al., 2000; Huang et al., 2005; Liang et al., 2005). Each studied group was injected subcutaneously near the abdominal midline of the mouse: one injection (0.5 ml) on each side.

The injected samples (Matrigel plugs) were retrieved on day 7 after implantation. At retrieval, the appearance of each test sample was grossly examined and photographed. Subsequently, the Matrigel plugs were retrieved and processed for histological examinations or for the determination of hemoglobin contents.

2.5. Histological examinations

In histological examinations, the fixed samples were embedded in paraffin, sectioned into a thickness of 5 μm, and then stained with hematoxylin and eosin (H&E). The stained sections of each test sample then were examined using light microscopy (Nikon Microphoto-FXA). The density of neo-vessels (angiogenesis) infiltrated into each studied sample was quantified with a computer-based image analysis system (Image-Pro® Plus) at a ×200 magnification (Bader et al., 1998). A minimum of five fields was counted for each retrieved sample. Additional sections were stained for factor VIII with immunohistological technique with a monoclonal anti-factor VIII antibody (Dako Co., Carpinteria, CA, USA).

The extent of vascularization in each retrieved sample was determined by measuring the amount of hemoglobin (Tabata et al., 1999). Test samples were fragmented with a scalpel and immersed into 17 mM of Tris–HCl buffer solution (pH 7.6) containing 0.75% of ammonium chloride for 24 h at 4 °C to extract hemoglobin in test samples. The extracted hemoglobin was quantified using a hemoglobin assay kit (Wako, Osaka, Japan).

2.6. Chemical stability

Rg_1 and Re were incubated in a sterile condition at 37, 50, or 100 °C overnight. Subsequently, each test solution was analyzed by a high-performance liquid chromatographer (HPLC) equipped with a C18 column (250 mm × 4.6 mm, BDS HYPERSIL, Thermo Electron Corp., Hercules, CA, $n = 5$; Caccia et al., 1992).

2.7. Statistical analysis

Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, which was performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC, USA). All data are presented as mean values ± S.D.

3. Results and discussion

bFGF is one of the endogenous angiogenic factors which stimulates proliferation and migration of endothelial cells (Nehls et al., 1994) and was reported to have various biological activities available to tissue regeneration and tissue engineering (Lee et al., 2000). It is weakly stable once the chemical or physical conditions of its exposed medium have been changed. Consequently, heparin is required for bFGF storage (Zamai et al., 1998). Binding of bFGF to heparin induces a conformational change in the bFGF molecule, resulting in an increased resistance against thermal denaturation and enzymatic degradation, and a reduced inactivation at acidic pH (Wissink et al., 2000). Thus, most of the commercially available bFGF compounds are heparin protected (Zamai et al., 1998).

3.1. HUVEC proliferation assay

The results of effects of bFGF, Rg_1, and Re, before (fresh) and after being treated at various temperatures for distinct durations, on HUVEC proliferation are shown in Table 1. The activity of each test agent on HUVEC proliferation, estimated by the MTS assay (Sakamoto et al., 2000; Terai et al., 2001), was expressed as the percentage of the sample absorbance to that of untreated control cells (the blank control in which no supplemented test agent was added). As shown, for fresh bFGF, Rg_1, and Re, HUVEC proliferations were increased 35, 17, and 17% of untreated control cells, respectively, which were found to be significant ($P < 0.05$). After being treated at different temperatures, the remaining activity of bFGF on HUVEC proliferation reduced significantly ($P < 0.05$). This observation was more significant with increasing the temperature treated ($P < 0.05$) and the duration of treatment ($P < 0.05$). In contrast, temperature did not seem to significantly affect the activities of Rg_1 and Re on

Table 1
Effects of bFGF and ginsenoside Rg_1 (Rg_1) and Re (Re), before (fresh) and after being treated at various temperatures, pHs, and solvent species for distinct durations, on HUVEC proliferation ($n = 5$)

Effect of temperature

	Test agent (%)								
	bFGF (fresh = 135.5 ± 0.4%)			Rg_1 (fresh = 117.8 ± 0.5%)			Re (fresh = 116.9 ± 0.9%)		
Temperature (°C)	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
4	126.1 ± 0.6	122.9 ± 0.4	119.5 ± 1.6	117.2 ± 0.6	116.6 ± 0.4	117.0 ± 1.4	116.7 ± 0.6	117.0 ± 0.5	116.6 ± 2.5
25	120.8 ± 0.7	116.1 ± 0.4	112.2 ± 1.5	117.1 ± 0.6	116.8 ± 0.5	115.4 ± 2.6	116.9 ± 0.2	116.4 ± 0.6	117.4 ± 0.4
37	117.9 ± 0.7	112.3 ± 0.6	107.4 ± 1.1	117.1 ± 0.7	117.3 ± 0.5	116.3 ± 2.7	116.9 ± 0.9	116.3 ± 0.7	117.4 ± 2.5
50	113.7 ± 0.7	107.9 ± 1.2	101.9 ± 0.3	117.1 ± 0.8	116.9 ± 0.6	117.3 ± 1.5	116.6 ± 1.1	116.6 ± 0.6	117.2 ± 1.9

Effect of pH

	Test agent (%)								
	bFGF			Rg_1			Re		
pH	IAT[a]	Day 3	Day 7	IAT[a]	Day 3	Day 7	IAT[a]	Day 3	Day 7
4.0	114.5 ± 0.5	111.3 ± 0.3	108.6 ± 0.2	115.2 ± 0.3	115.4 ± 0.6	115.1 ± 1.1	115.5 ± 0.7	115.4 ± 0.5	115.7 ± 0.1
7.4	131.8 ± 0.6	118.2 ± 0.4	111.5 ± 0.8	115.5 ± 0.1	115.5 ± 0.1	115.7 ± 1.1	115.3 ± 0.6	115.7 ± 1.1	115.9 ± 0.7
10.0	113.4 ± 0.4	111.4 ± 0.6	108.9 ± 0.3	115.6 ± 0.7	115.3 ± 0.4	115.3 ± 0.7	115.2 ± 0.6	115.3 ± 0.4	115.0 ± 0.4

Effect of solvent species

	Test agent (%)								
	bFGF			Rg_1			Re		
Solvent	IAT[a]	Day 3	Day 7	IAT[a]	Day 3	Day 7	IAT[a]	Day 3	Day 7
20% Ethanol	134.9 ± 1.0	121.2 ± 0.6	107.9 ± 0.5	117.2 ± 0.9	116.9 ± 0.7	117.8 ± 0.8	117.6 ± 1.1	117.1 ± 0.5	117.2 ± 1.2
Ethanol	135.3 ± 0.6	118.1 ± 0.9	106.0 ± 0.9	117.2 ± 0.9	117.3 ± 0.7	117.4 ± 0.5	117.1 ± 0.6	117.2 ± 0.6	117.2 ± 0.8
DMSO[b]	129.6 ± 0.4	120.9 ± 0.5	107.6 ± 1.5	117.2 ± 0.4	116.9 ± 1.9	117.2 ± 1.3	117.1 ± 1.0	117.5 ± 1.5	117.6 ± 1.8

The activity of each test agent on HUVEC proliferation, estimated by the MTS assay, was expressed as the percentage of the sample absorbance to that of untreated control cells.
[a] IAT: immediately after treatment.
[b] DMSO: dimethyl sulfoxide.

HUVEC proliferation throughout the entire course of the study ($P > 0.05$, Table 1).

After being treated at different pHs for distinct durations, the remaining activity of bFGF on HUVEC proliferation decreased significantly, particularly at pH 4.0 and 10.0. As indicated in Table 1, the activity of bFGF reduced rapidly immediately after being exposed at pH 4.0 or 10.0, while that treated at pH 7.4 decreased gradually ($P < 0.05$). It was reported that bFGF is most thermally stable between pH 7.0 and 9.0. Samples of bFGF in the pH range of 2.0–4.0 showed several faint bands of protein on SDS-PAGE gel which is an indication of protein fragmentation (Vemuri et al., 1994). In contrast, the activities of Rg_1 and Re on HUVEC proliferation stayed approximately the same ($P > 0.05$). After being treated in various solvent species, the activity of bFGF on HUVEC proliferation also declined with time ($P < 0.05$, Table 1), while the activities of Rg_1 and Re remained unchanged throughout the entire course of the study ($P > 0.05$).

The aforementioned results indicated that changes in the chemical or physical parameters such as pH, temperature, or solvent species could significantly affect the activity of bFGF on HUVEC proliferation, even though it was heparin protected. In contrast, the activities of Rg_1 and Re on HUVEC proliferation remained approximately the same. It is known that proteins are often unstable without being in their native environments. The occurrence of protein denaturation may be attributed to a variety of factors, including changes in pH, buffer species, and temperature, which in turn may cause conformation changes and protein precipitation (Fransson et al., 1997; Shah et al., 1998; Vemuri et al., 1994). It was shown that bFGF is uniquely susceptible to acid and heat treatments (Ogura et al., 1999). Even if at physiological pH and temperature, the *in vitro* half-lifetime of bFGF is approximately 12 h (Perets et al., 2003; Westal et al., 1983).

Additionally, proteins are unstable in most polar solvents such as ethanol and DMSO (Pace et al., 2004). Previous reports indicated that DMSO is a protein-dissolving solvent and is capable of causing partial unfolding or denaturation of proteins (Zheng and Ornstein, 1996). On the contrary, ginsenosides such as Rg_1 and Re share a similar basic structure, consisting of gonane steroid nucleus having 17 carbon atoms arranged in four rings (Popovich and Kitts, 2002). These specific chemical structures consist of a high degree of ring structure (Fig. 1), which makes their flexibility very limited. The ring structures are known to be inherently stable (Tongay et al., 2005).

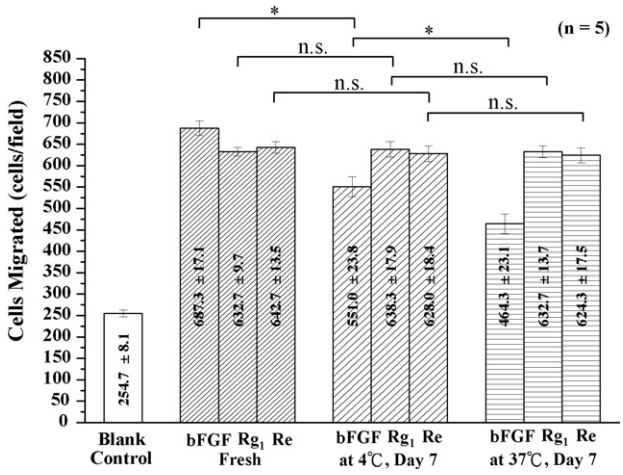

Fig. 2. Effects of bFGF and ginsenoside Rg_1 (Rg_1) and Re, before (fresh) and after being treating at 4 or 37 °C for 7 days, on HUVEC migration obtained in a Transwell-plate assay, n.s. indicates no statistical difference; *indicates statistical significance at a level of $p < 0.05$.

The activities of bFGF, Rg_1, and Re before and after being treated at 4 or 37 °C for 7 days on HUVEC migration and tube formation were further evaluated *in vitro*.

3.2. HUVEC migration assay

HUVEC migration assays were conducted in Transwell plates (Facchiano et al., 2002) using Matrigel-coated membranes. Cells were added to the upper chamber and after 12 h, the number of cells migrated through the membrane in response to each test agent in the lower chamber was quantified. As shown in Fig. 2, HUVECs treated with fresh bFGF, Rg_1, and Re showed approximately 2.7, 2.5, and 2.5 times of the migratory activity of untreated control cells, respectively ($P < 0.05$). However, after being treated, the remaining activity of bFGF on HUVEC migration reduced significantly ($P < 0.05$), while those of Rg_1 and Re remained unchanged ($P > 0.05$).

3.3. HUVEC tube formation assay

Enhancements of HUVEC migration by bFGF, Rg_1, and Re suggested that these test agents might also enhance HUVEC tube formation *in vitro* (Hotchkiss et al., 2002). As shown in Fig. 3a, when placed on Matrigel in the absence of test agents (the blank control), HUVECs formed incomplete and narrow tube-like structures. In contrast, following stimulation with bFGF, Rg_1, or Re, formation of elongated and robust tube-like structures was observed which were organized by a greater number of cells as compared to the blank control. The ability of HUVECs to form a network of tubular structures across the surface of a Matrigel substratum is a complex phenomenon that combines elements of attachment, migration, organization, and differentiation (Grant et al., 1991). Other cell types such as salivary gland, mammary, renal tubular, or bone cells also exhibit organization on Matrigel (Kibbey et al., 1992; Seely and Aggeler, 1991). The complex organizational behavior of HUVECs on Matrigel models the type of coordinated activities required for angiogenesis by endothelial cells (Morales et al., 1995).

The observed tube formation was further quantitatively estimated by measuring the area covered by the tube network using an image analysis system. Tube formation activity was expressed as the percentage of the area to that of the blank control. Fig. 3b shows that fresh bFGF, Rg_1, and Re stimulated HUVEC tube formation, and this stimulation was found to be statistically significant as compared to the blank control ($P < 0.05$). However, after being treated, the remaining activity of bFGF in stimulating HUVEC tube formation reduced significantly ($P < 0.05$), while those of Rg_1 and Re remained approximately the same ($P > 0.05$).

The aforementioned results indicated that bFGF, Rg_1, and Re enhanced several *in vitro* HUVEC activities related to angiogenesis, including proliferation, migration, and tube formation. However, the activity of bFGF was significantly affected by the exposure to temperature, pH, and solvent species, while those of Rg_1 and Re remained unchanged. To further determine whether bFGF, Rg_1, and Re before and after being treated at 37 °C for 7 days were capable of stimulating angiogenesis *in vivo*, an established *in vivo* angiogenesis model, the mouse Matrigel plug assay (Sengupta et al., 2004), was performed.

3.4. Chemical stability

Results of our HPLC analyses showed that there were no significant changes in the chromatograms of Rg_1 and Re before incubation and those incubated at 37, 50, or 100 °C overnight. In contrast, it was reported that incubation of bFGF at the same conditions resulted in a loss of its chemical stability as compared to its starting HPLC chromatogram (Caccia et al., 1992). These results indicated that the chemical stability of Rg_1 and Re was significantly better than that of bFGF.

3.5. In vivo study

Matrigel alone (MG/Control) or containing each test agent were subcutaneously injected into C57/BL6 mice, and 7 days later, the formed Matrigel plugs in mice were photographed and excised. Fig. 4 illustrates representative photographs of the MG/Control, MG/bFGF, MG/Rg_1, MG/Re, MG/bFGF-Day 7, MG/Rg_1-Day 7, and MG/Re-Day 7 plugs observed on day 7 after implantation. As shown, the plug with Matrigel alone (MG/Control) was clear in its color, indicating no or less blood vessel formation. In contrast, Matrigel plugs mixed with fresh (MG/bFGF, MG/Rg_1l, MG/Re) or treated (MG/bFGF-Day 7, MG/Rg_1-Day 7, and MG/Re-Day 7) test agents appeared red color.

In accordance with the gross appearance, histological examinations of the red areas in the test groups showed numerous vascular structures with cells infiltrated (Fig. 5). These vessels abundantly filled with red blood cells, which indicated the formation of a functional vasculature inside the Matrigel plugs. The in-growing vessels were coated with an inner endothelial layer staining positive with a monoclonal antibody against factor VIII.

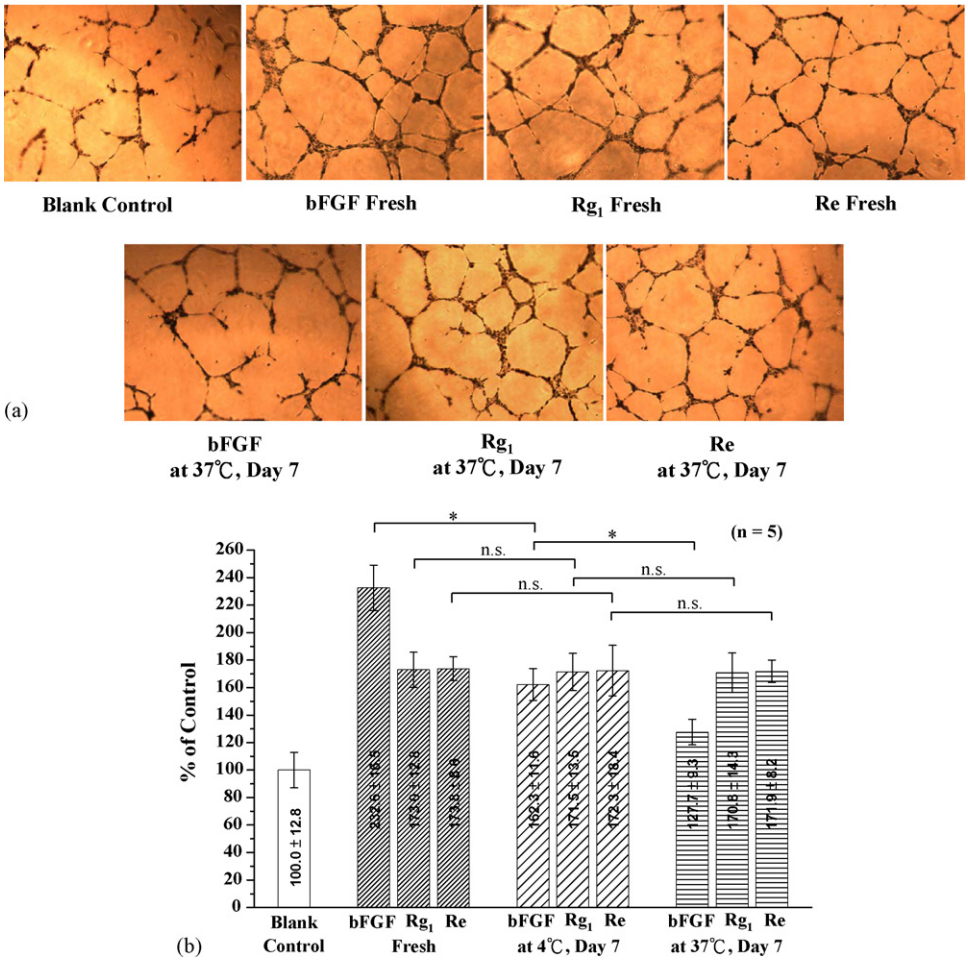

Fig. 3. (a) Photomicrographs showing effects of bFGF and ginsenoside Rg_1 (Rg_1) and Re, before (fresh) and after being treated at 37 °C for 7 days, on tube formation by HUVECs (magnification, ×40). (b) Quantification of tube formation in the presence or absence of bFGF or ginsenoside Rg_1 (Rg_1) or Re (Re), before (fresh) and after being treating at 4 or 37 °C for 7 days. Tube formation was quantitatively estimated by measuring the area covered by the tube network using an image analysis program, n.s. indicates no statistical difference; *indicates statistical significance at a level of $p < 0.05$.

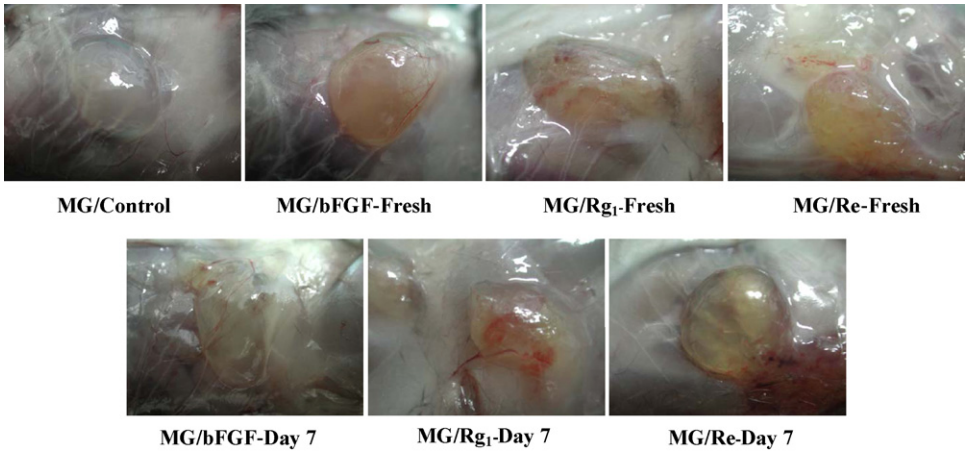

Fig. 4. Photographs of all implanted samples observed on day 7 after implantation. MG/Control: Matrigel alone; MG/bFGF-Fresh, MG/Rg_1-Fresh, and MG/Re-Fresh: Matrigel mixed with fresh bFGF, Rg_1, and Re, respectively; MG/bFGF-Day 7, MG/Rg_1-Day 7, and MG/Re-Day 7: Matrigel mixed with bFGF, Rg_1, and Re, respectively, after being treated at 37 °C for 7 days.

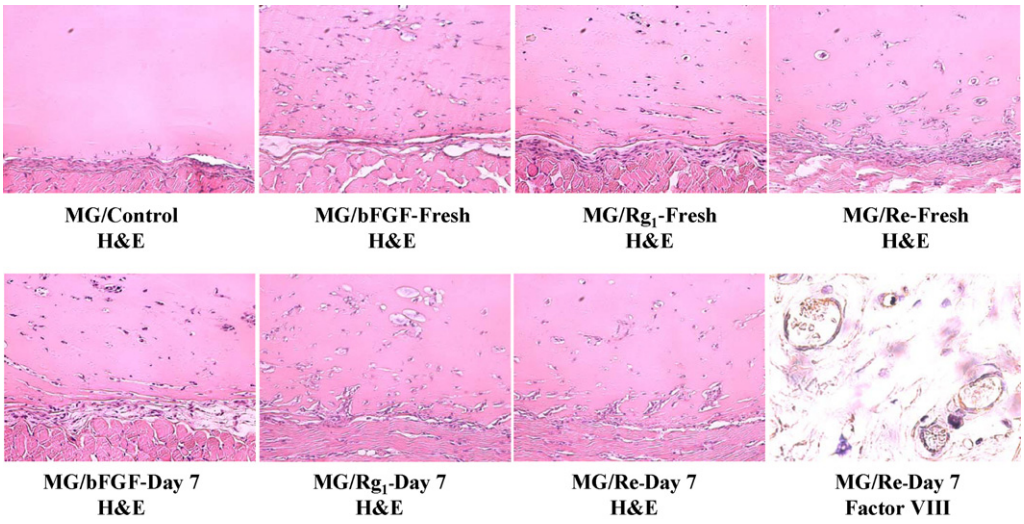

Fig. 5. Photomicrographs of all test samples retrieved on day 7 after implantation showing cell invasion with functional capillaries with red blood cells in the lumen. H&E: stained with hematoxylin and eosin (magnification, ×200). Factor VIII: stained with a monoclonal antibody against factor VIII (magnification, ×800).

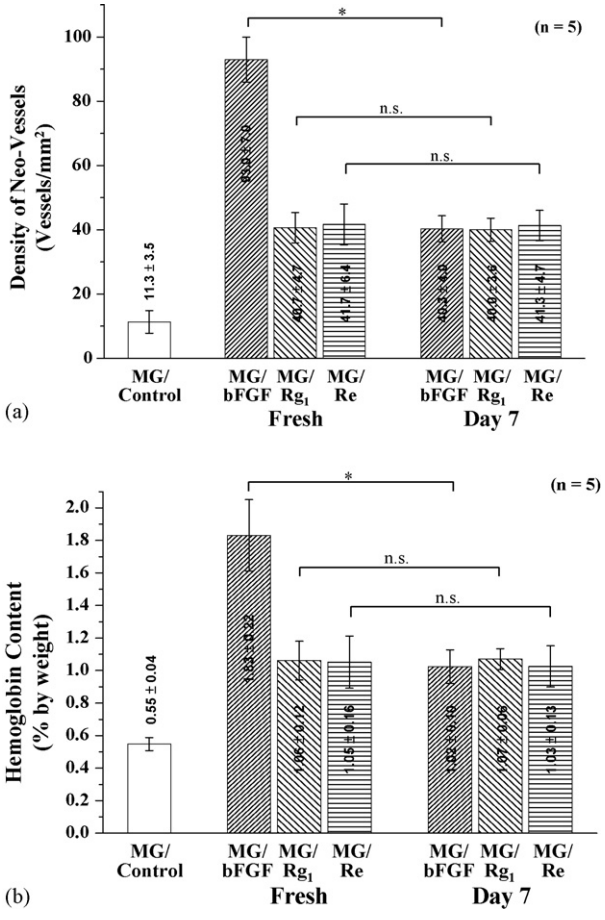

Fig. 6. Quantitative analyses of: (a) the density of neo-vessels and (b) the tissue hemoglobin content observed in each test group observed on day 7 after implantation. n.s. indicates no statistical difference; *indicates statistical significance at a level of $p < 0.05$.

In contrast, the Matrigel alone group (MG/Control) was slightly vascularized.

The density of neo-vessels infiltrated into the Matigel plugs mixed with fresh bFGF, Rg_1, and Re (MG/bFGF, MG/Rg_1, and MG/Re) were significantly greater than the Matrigel alone group (MG/Control, $P < 0.05$, Fig. 6a). However, the density of neo-vessels was significantly reduced in the Matrigel plug mixed with bFGF after being treated at 37 °C for 7 days (MG/bFGF-Day 7). In contrast, its Rg_1 and Re counterparts (MG/Rg_1-Day 7 and MG/Re-Day 7) remained unchanged ($P > 0.05$). These results were further confirmed by the determination of hemoglobin contents in the Matrigel plugs (Fig. 6b).

4. Conclusions

The results obtained in the study indicated that the angiogenic activity of bFGF was significantly affected by its environmental parameters such as temperature, pH, and solvent species, while those of Rg_1 and Re remained unchanged throughout the experimental period. These results suggested that Rg_1 and Re could be a novel group of nonpeptide angiogenic agents with a superior stability and may be used for the management of tissue regeneration.

Acknowledgments

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References

Babaei, S., Stewart, D.J., 2002. Overexpression of endothelial NO synthase induces angiogenesis in a co-culture model. Cardiovasc. Res. 55, 190–200.

Bader, A., Schiling, T., Teebken, O.E., 1998. Tissue engineering of heart valves-human endothelial cell seeding of detergent acellularized porcine valves. Eur. J. Cardiothoracic. Surg. 14, 279–284.

Caccia, P., Nitti, G., Cletini, O., Pucci, P., Ruoppolo, M., Bertolero, R., Valsasina, B., Roletto, E., Cristiani, C., Cauet, G., Sarmientos, P., Malorni, A., Marino, G., 1992. Stabilization of recombinant human basic fibroblast growth factor by chemical modifications of cycteine residues. Eur. J. Biochem. 204, 649–655.

Castro, C.A., Hogan, J.B., Benson, K.A., Shehata, C.W., Landauer, M.R., 1995. Behavioral effects of vehicles: DMSO, ethanol, Tween-20, Tween-80, and emuiphor-620. Pharmacol. Biochem. Behav. 50, 521–526.

Chan, R.Y.K., Chen, W.F., Dong, A., Guo, D., Wong, M.S., 2002. Estrogen-like activity of ginsenoside-Rg_1 derived from panax notoginseng. J. Clin. Endocrinol. Metab. 87, 3691–3695.

Facchiano, F., Lentini, A., Fogliano, V., Mancarella, S., Rossi, C., Facchiano, A., Capogrossi, M.C., 2002. Sugar-induced modification of fibroblast growth factor 2 reduces its angiogenic activity *in vivo*. Am. J. Pathol. 161, 531–541.

Fransson, J., Hallen, D., Florin-Robertsson, E., 1997. Solvent effects on the solubility and physical stability of human insulin-like growth factor I. Pharm. Res. 14, 606–612.

Grant, D.S., Lelkes, P.L., Fukuda, K., Kleinman, H.K., 1991. Intracellular mechanisms involved in basement membrane induced blood vessel differentiation *in vitro*. In Vitro Cell Dev. Biol. 27A, 327–336.

Hall, H., Baechi, T., Hubbell, J.A., 2001. Molecular properties of fibrin-based matrices for promotion of angiogenesis *in vitro*. Microvasc. Res. 62, 315–326.

Hotchkiss, K.A., Ashton, A.W., Mahmood, R., Russell, R.G., Sparano, J.A., Schwartz, E.L., 2002. Inhibition of endothelial cell function *in vitro* and angiogenesis *in vivo* by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol. Cancer Ther. 1, 1191–1200.

Huang, Y.C., Chen, C.T., Chen, S.C., Lai, P.H., Liang, H.C., Chang, Y., Yu, L.C., Sung, H.W., 2005. A natural compound (ginsenoside Re) isolated from *Panax ginseng* as a novel angiogenic agent for tissue regeneration. Pharm. Res. 22, 636–646.

Kelm, J.M., Ehler, E., Nielsen, L.K., Schlatter, S., Perriard, J.C., Fussenegger, M., 2004. Design of artificial myocardial nicrotissues. Tissue Eng. 10, 201–214.

Kibbey, M.C., Royce, L.S., Dym, M.S., Baum, B.J., Kleinman, H.K., 1992. Glandular morphogenesis of a human submandibular cell line by basement membrane components *in vitro*. Exp. Cell Res. 198, 343–357.

Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D.J., Bohlen, P., Kerbel, R.S., 2000. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Invest. 105, 15–24.

Lee, K.Y., Halberstadt, C.R., Holder, W.D., Mooney, D.J., 2000. Breast reconstruction. In: Lanza, R.P., Langer, R., Vacanti, J. (Eds.), Principles of Tissue Engineering. Academic Press, New York, pp. 409–423.

Lee, Y.J., Chung, E., Lee, K.Y., Lee, Y.H., Huh, B., Lee, S.K., 1997. Ginsenoside-Rg_1, one of the major active molecules from *Panax ginseng*, is a functional ligand of glucocorticoid receptor. Mol. Cell. Endocrinol. 133, 135–140.

Ley, C.D., Olsen, M.W.B., Lund, E.L., Kristjansen, P.E.G., 2004. Angiogenic synergy of bFGF and VEGF is antagonized by angiopoietin-2 in a modified *in vivo* matrigel assay. Microvasc. Res. 68, 161–168.

Liang, H.C., Chen, C.T., Chang, Y., Huang, Y.C., Chen, S.C., Sung, H.W., 2005. Loading of a novel angiogenic agent, ginsenoside Rg_1 in an acellular biological tissue for tissue regeneration. Tissue Eng. 11, 835–846.

Morales, D.E., McGowan, K.A., Grant, D.S., Maheshwari, S., Bhartiya, D., Cid, M.C., Kleinman, H.K., Schnaper, H.W., 1995. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells *in vitro* and in a murine model. Circulation 91, 755–763.

Morisaki, N., Watanabe, S., Tezuka, M., Zenibayashi, M., Shiina, R., Koyama, N., Kanzaki, T., Saito, Y., 1995. Mechanism of angiogenic effects of saponin from Ginseng Radix rubra in human umbilical vein endothelial cells. Br. J. Pharmacol. 115, 1188–1193.

Nehls, V., Schuchardt, E., Drenckhahn, D., 1994. The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. Microvasc. Res. 48, 349–363.

Nie, D., Lamberti, M., Zacharek, A., Li, L., Szekeres, K., Tang, K., Chen, Y.C., Honn, K.V., 2000. Thromboxane A2 regulation of endothelial cell migration, angiogenesis, and tumor metastasis. Biochem. Biophys. Res. Commun. 267, 245–251.

Ogura, K., Nagata, K., Hatanaka, H., Habuchi, H., Kimata, K., Tate, S., Ravera, M.W., Jaye, M., Schlessinger, J., Inagaki, E., 1999. Solution structure of human acidic fibroblast growth factor and interaction with heparin-derived hexasaccharide. J. Biomol. NMR 13, 11–24.

Pace, C.N., Trevino, S., Prabhakaran, E., Scholtz, J.M., 2004. Protein structure, stability and solubility in water and other solvents. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1225–1235.

Perets, A., Baruch, Y., Weisbuch, R., Shoshany, G., Neufeld, G., Cohen, S., 2003. Enhancing the vascularization of three-dimensional porous alginate scaffolds by incorporating controlled release basic fibroblast growth factor microspheres. J. Biomed. Mater. Res. 65A, 489–497.

Pieper, J.S., Hafmans, T., van Wachem, P.B., van Luyn, M.J.A., Brouwer, L.A., Veerkamp, J.H., van Kuppevelt, T.H., 2002. Loading of collagen-heparin sulfate matrices with bFGF promotes angiogenesis and tissue generation in rats. J. Biomed. Mater. Res. 62, 185–194.

Popovich, D.G., Kitts, D.D., 2002. Structure-function relationship exists for ginsenosides in reducing proliferation and inducing apoptosis in the human leukemia (THP-1) cell line. Arch. Biochem. Biophys. 406, 1–8.

Prisell, P.T., Camber, O., Hiselius, J., Norstedt, G., 1992. Evaluation of hyaluronan as a vehicle for peptide growth factors. Int. J. Pharm. 85, 51–56.

Sakamoto, H., Mashima, T., Kizaki, A., Dan, S., Hashimoto, Y., Naito, M., Tsuruo, T., 2000. Glyoxalase I is involved in resistance of human leukemia cells to antitumor agent-induced apoptosis. Blood 95, 3214–3218.

Scott, G.I., Colligan, P.B., Ren, B.H., Ren, J., 2001. Ginsenosides Rb_1 and Re decrease cardiac contraction in adult rat ventricular myocytes: role of nitric oxide. Br. J. Pharmacol. 134, 1159–1165.

Seely, K.A., Aggeler, J., 1991. Modulation of milk protein synthesus through alteration of the cytoskeleton in mouse mammary epithelial cells cultured on a reconstituted basement membrane. J. Cell Physiol. 146, 117–130.

Sengupta, S., Toh, S.A., Sellers, L.A., Skepper, J.N., Koolwijk, P., Leung, H.W., Yeung, H.W., Wong, R.N.S., Sasisekharan, R., Fan, T.P.D., 2004. Modulating angiogenesis: the yin and the yang in ginseng. Circulation 110, 1219–1225.

Shah, D., Johnston, T.P., Mitra, A.K., 1998. Thermodynamic parameters associated with guanidine HCl- and temperature-induced unfolding of bFGF. Int. J. Pharm. 169, 1–14.

Shea, L.D., Smiley, E., Bonadio, J., Mooney, D.J., 1999. DNA delivery from polymer matrices for tissue engineering. Nat. Biotechnol. 17, 551–554.

Tabata, Y., Miyao, M., Yamamoto, M., Ikada, Y., 1999. Vascularization into a porous sponge by sustained release of basic fibroblast growth factor. J. Biomater. Sci. Polym. Ed. 10, 957–968.

Tabata, Y., 2003. Tissue regeneration based on growth factor release. Tissue Eng. 9, S5–S15.

Terai, Y., Abe, M., Miyamoto, K., Koike, M., Yamasaki, M., Ueda, M., Ueki, M., Sato, Y., 2001. Vascular smooth muscle cell growth-promoting factor/F-Spondin inhibits angiogenesis via the blockade of integrin $\alpha v\beta 3$ on vascular endothelial cells. J. Cell. Physiol. 188, 394–402.

Tongay, S., Dag, S., Durgun, E., Senger, R.T., Ciraci, S., 2005. Atomic and electronic structure of carbon strings. J. Phys.: Condens. Matter 17, 3823–3836.

Vemuri, S., Beylin, I., Sluzky, V., Stratton, P., Eberlein, G., Wang, Y.J., 1994. The stability of bFGF against thermal denaturation. J. Pharm. Pharmacol. 46, 481–486.

Wang, W., 2005. Protein aggregation and its inhibition in biopharmaceutics. Int. J. Pharm. 289, 1–30.

Westal, F.C., Rubin, R., Gospodarowicz, D., 1983. Brain derived fibroblast growth factor: a study of its inactivation. Life Sci. 33, 2425–2429.

Wissink, M.J.B., Beernink, R., Poot, A.A., Engbers, G.H.M., Beugeling, T., van Aken, W.G., Feijen, J., 2000. Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices. J. Control. Release 64, 103–114.

Zamai, M., Caiolfa, V.R., Pines, D., Parola, A.H., 1998. Nature of interaction between basic fibroblast growth factor and the antiangiogenic drug 7,7-(car-

bonyl-bis[imino-*N*-methyl-4,2-pyrrolecarbonylimino[*N*-methyl-4,2-pyrrole]-c arbonylimino])bis-(1,3-naphthalene disulfonate). Biophys. J. 75, 672–682.

Zhang, Y.W., Dou, D.Q., Zhang, L., Chen, Y.J., Yao, X.S., 2001. Effects of ginsenosides from panax ginseng on cell-to-cell communication function mediated by gap junctions. Planta Med. 67, 417–422.

Zheng, Y.J., Ornstein, R.L., 1996. A molecular dynamics and quantum mechanics analysis of the effect of DMSO on enzyme structure and dynamics: subtilisin. J. Am. Chem. Soc. 118, 4175–4180.

Zour, E., Lodhl, S.A., Nesbitt, R.U., Sllbering, S.B., Chaturvedl, P.R., 1992. Stability studies of gabapentin in aqueous solutions. Pharm. Res. 9, 595–600.