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Research Paper

A Natural Compound (Ginsenoside Re) Isolated from *Panax ginseng* as a Novel Angiogenic Agent for Tissue Regeneration

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Purpose. The primary challenge for tissue engineering is to develop a vascular supply that can support the metabolic needs of the engineered tissues in an extracellular matrix. In this study, the feasibility of using a natural compound, ginsenoside Re, isolated from *Panax ginseng* in stimulating angiogenesis and for tissue regeneration was evaluated.

Methods. Effects of ginsenoside Re on the proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) were examined *in vitro*. Additionally, angiogenesis and tissue regeneration in a genipin-fixed porous acellular bovine pericardium (extracellular matrix; ECM) incorporated with ginsenoside Re implanted subcutaneously in a rat model were investigated. Basic fibroblast growth factor (bFGF) was used as a control.

Results. It was found that HUVEC proliferation, migration in a Transwell plate, and tube formation on Matrigel were all significantly enhanced in the presence of bFGF or ginsenoside Re. Additionally, effects of ginsenoside Re on HUVEC proliferation, migration, and tube formation were dose-dependent and reached a maximal level at a concentration of about 30 μg/ml. The *in vivo* results obtained at 1 week postoperatively showed that the density of neocapillaries and the tissue hemoglobin content in the ECMs were significantly enhanced by bFGF or ginsenoside Re. These results indicated that angiogenesis in the ECMs was significantly enhanced by loading with bFGF or ginsenoside Re. At 1 month postoperatively, vascularzied neo-connective-tissue fibrils were found to fill the pores in the ECMs loaded with bFGF or ginsenoside Re.

Conclusions. The aforementioned results indicated that like bFGF, ginsenoside Re-associated induction of angiogenesis enhanced tissue regeneration, supporting the concept of therapeutic angiogenesis in tissue-engineering strategies.

KEY WORDS: acellular tissue; angiogenic agent; ginsenoside Re; tissue regeneration.

INTRODUCTION

The primary challenge for tissue engineering is to develop a vascular supply that can support the metabolic needs of the engineered or regenerated tissues in extracellular matrices (1). It is known that oxygen supply is often limited in a tissue-engineered extracellular matrix (ECM). Cells more than approximately 200 μm from a blood vessel suffer from hypoxia and die (2,3). In tissue repair or regeneration, the vasculature of the host penetrates macroporous matrices used for cell delivery via the process of angiogenesis, by which new blood vessels sprout from those that already exist and grow into undervascularized regions nearby (4). Investigations have incorporated angiogenic factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) to stimulate angiogenesis in tissue-engineered extracellular matrices. However, the biological activity of protein-type growth factors may not last long *in vivo* because of their poor stability (5,6). It was demonstrated that the loss of the biological activity results from denaturation and deactivation of proteins during the formulation process with a polymeric matrix (7).

Panax ginseng has long been used in herbal medicine. It has multiple pharmacological actions and has been used for the treatments of cardiovascular diseases and cancers as well as in improving overall stress tolerance (8,9) and in the repair of intractable skin ulcers of patients with diabetes mellitus (10). Angiogenesis is known to play an important role in the

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; ECM, extracellular matrix; ECM/bFGF, the ECM loaded with bFGF; ECM/control, the ECM dip-coated in the drug-free gelatin solution; ECM/Re, the ECM loaded with ginsenoside Re; H&E, hematoxylin and eosin; HUVEC, human umbilical vein endothelial cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline.

repair of ulcers. The major active component of ginseng is saponin, and it includes more than 20 identified ginsenosides (10). Effects of saponin isolated from ginseng (*Ginseng radix rubra*) on angiogenesis (human umbilical vein endothelial cell, HUVEC, tube formation) were previously examined *in vitro* by Morisaki *et al.* (10). Their results indicated that saponin stimulates human umbilical vein endothelial cell (HUVEC) tube formation mainly through modification of the balance of protease/protease inhibitor secreted from HUVECs.

Ginsenoside Re is one of the major active components of saponin in *Panax ginseng* (11). Experimentally, ginsenoside Re was shown to markedly depress the P wave in electrocardiogram, dilate the canine vessels, and significantly stimulate the activity of nitric oxide synthase (NOS) (11–13). It was reported that nitric oxide (NO) is a downstream mediator in the angiogenic response to a variety of growth factors (14). It is, therefore, speculated that ginsenoside Re may be used to stimulate angiogenesis and can be incorporated in an extracellular matrix for tissue regeneration.

In this study, effects of ginsenoside Re on HUVEC proliferation, migration, and tube formation were examined *in vitro*. Additionally, angiogenesis and tissue regeneration in a genipin-fixed porous acellular bovine pericardium incorporated with ginsenoside Re implanted subcutaneously in a rat model were investigated. It was found in our previous study that acellular bovine pericardia fixed with genipin can provide a natural microenvironment for host cell migration and proliferation and may be used as a tissue-engineering extracellular matrix to accelerate tissue regeneration (15). bFGF, a commonly used angiogenic agent in tissue engineering, was used as a control.

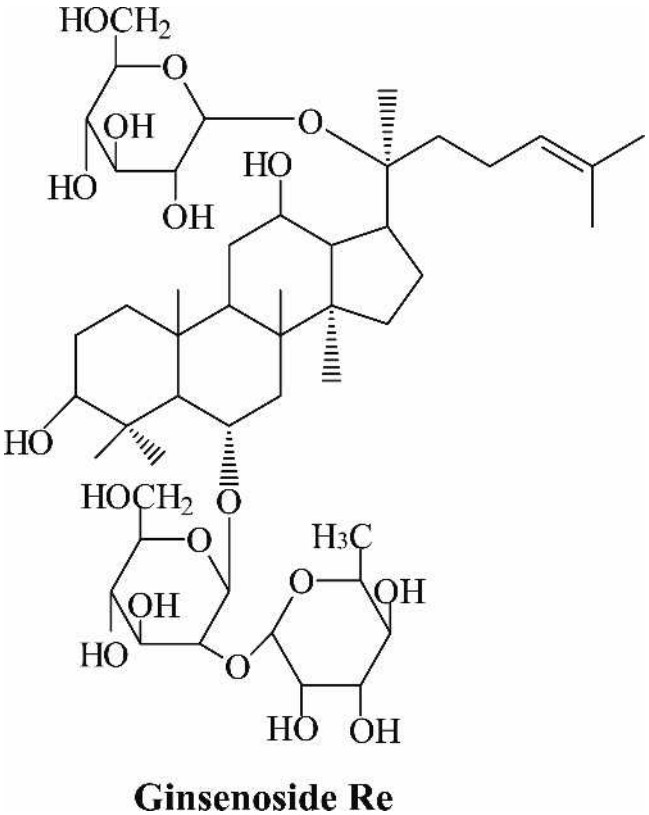

Fig. 1. Chemical structure of ginsenoside Re.

MATERIALS AND METHODS

Materials

Ginsenoside Re (Fig. 1) was purchased from Extrasynthese Co., Ltd. (Zone Industrielle, Lyon Nord, France). In the study, ginsenoside Re stocks of 2.5 mg/ml were prepared in 20% ethanol and used for all experiments. However, the final content of ethanol in the growth medium was less than 0.5% (v/v), which would not interfere with the test system. Additionally, all the control groups contained the same amount of ethanol as that in the compound-treated group.

In Vitro Study

The series of assays used in the *in vitro* study were designed to examine various aspects of angiogenic behavior of ginsenoside Re and have been widely used in the literature (16). HUVECs (Cascade Biologics, 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 Biologics). 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.

HUVEC Proliferation

HUVECs were cultured on collagen-coated 96-well plates at a density of 6000 cells/well for 24 h. Subsequently, the cells were treated with testing samples by replacing the media with 0.1 ml Medium 200 containing 2% fetal bovine serum (FBS, Cascade Biologics). The growth medium was supplemented with ginsenoside Re at varying concentrations. A positive control, in which 10 ng/ml bFGF (PeproTech, Rockhill, NJ, USA) was added, and a blank control, in which no supplemented ginsenoside Re or bFGF was added, were performed. After cells were incubated for 48 h, cell numbers were estimated by the MTS assay (17,18). Briefly, 0.1 ml 20% [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS; CellTiter 96 Aq_{ueous} One Solution Reagent, Promega, Madison, WI, USA] was added to each well and incubated at 37°C for a further 3 h. The absorbance of the formazan product was measured at 490 nm for each well using a microplate Reader (Model MRX, Dynatech Laboratories Inc., Chantilly, VA, USA).

HUVEC Migration

HUVEC migration assays were carried out in Transwell plates (COSTAR, Corning, NY, USA) (19). Confluent HUVEC monolayers were harvested with cell dissociation solution and suspended at 5×10^5 cells/ml in Medium 200 supplemented with 2% FBS. HUVECs (1×10^5) were placed in the upper chamber of the Transwell plate mounted with 8-μm pores polycarbonate filters coated with 10% Matrigel (Becton Dickinson Biosciences, San Jose, CA, USA). Ginsenoside Re was dissolved at varying concentrations in Medium 200 with 2% FBS and placed in the bottom chamber of the Transwell plate. The positive control chambers contained 10 ng/ml bFGF. Medium 200 plus 2% FBS was used as

the blank control. Migration assays were carried out at 37°C in 5% CO_2 for 12 h, and then filters were removed, fixed with 10% formalin, and stained with hematoxylin. Cells migrated were counted randomly with a Nikon-E-800, Tokyo, Japan, at 200× magnification in 6 fields for each filter, and the number of total fields was reported. Each data point was based on pentaplicate chambers (20).

HUVEC Tube Formation

HUVEC tube formation assays were performed using 96-well plates coated with 50 μl of Matrigel (Becton Dickinson Biosciences) per well (16). HUVECs were seeded on coated plates at 1×10^4 cells/well in Medium 200 containing 2% FBS. Samples of ginsenoside Re were tested at concentrations of 10 ng/ml to 100 μg/ml. bFGF (10 ng/ml) was tested as a positive control. Tubes were allowed to form by incubating 12 h at 37°C and the cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent (Sigma, St. Louis, MO, USA) and fixed with 10% formalin. Light micrographs of six microscopic fields (40× magnification) of the well were used for the assay of angiogenic activities by measuring the area covered by the tube network in the area photographed. The area covered by the tube network was quantified using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) (21). Each condition was assayed in pentaplicate wells (21,22).

Preparation of Extracellular Matrices

Bovine pericardia procured from a slaughterhouse were used as raw materials. The procedures used to remove the cellular components from bovine pericardia were based on a method previously reported by Courtman *et al.* with slight modifications (23). To increase the pore size and porosity within test samples, the acellular tissues were treated additionally with acetic acid and subsequently with collagenase (24). Finally, the acellular tissues with a porous structure were fixed in a 0.05% genipin (Challenge Bioproducts, Taichung, Taiwan) aqueous solution (pH 7.4) at 37°C for 3 days. The chemical structure of genipin can be found in the literature (25).

The degree of cross-linking of the genipin-fixed porous acellular bovine pericardium (used as an ECM in the study) was determined by measuring its fixation index and denaturation temperature (n = 5). The fixation index, determined by the ninhydrin assay, was defined as the percentage of free amino groups in test tissues reacted with genipin subsequent to fixation. The denaturation temperature of the test ECM was measured by a Perkin-Elmer differential scanning calorimeter (model DSC-7; Norwalk, CT, USA). Details of the methods used in the determinations of fixation index and denaturation temperature of test samples were previously described (25).

After preparation, the test ECM was processed for light microscopic examinations to investigate its ultrastructures. The pore size of the test ECM, stained with hematoxylin and eosin (H&E), was determined under a microscope (n = 5). The porosity of the test ECM was measured by helium pycnometery (n = 5) (26). The prepared ECM was sterilized in a graded series of ethanol solutions. Finally, the sterilized ECMs were rinsed in sterilized phosphate buffered saline (PBS).

To incorporate bFGF (0.7 μg, ECM/bFGF) or ginsenoside Re (70 μg, ECM/Re) in the ECMs (~10 × 10 mm), bFGF or ginsenoside Re was dissolved in a sterilized gelatin (300 mg/ml PBS, from bovine skin, 225 Bloom, Sigma) aqueous solution. The amount of bFGF loaded in the ECM was similar to that reported in the literature (27). In our preliminary study, angiogenesis in the ECM loaded with 0.7 μg ginsenoside Re was not apparent. Therefore, the amount of ginsenoside Re loaded in the ECM was increased to 70 μg. After vigorous mixing of the solution, the prepared ECMs were dip-coated in the drug-containing gelatin solution under a reduced pressure environment and subsequently gelled in liquid nitrogen. The ECM dip-coated in the drug-free gelatin solution (ECM/control) was used as a blank control. All experimental processes were conducted under sterile conditions.

In Vivo Study

The animal study was conducted under aseptic conditions using a rat model (4-week-old male Wistar). In total, 30 rats divided into three groups (ECM/control, ECM/bFGF, and ECM/Re) were used in the study. Rats were anesthetized by intramuscular injection of sodium pentobarbital (30 mg/ml). Two test samples of the same type were separately implanted subcutaneously in each rat. The implanted samples were retrieved at 1 week and 1 month (n = 5 at each time point) postoperatively. At retrieval, the appearance of each retrieved sample was examined grossly and photographed. Subsequently, one half of each retrieved sample was fixed and embedded in paraffin for histologic examinations, and the remainder was used to quantify the amount of tissue hemoglobin and to measure denaturation temperature.

Light Microscopic Examinations

In histologic examinations, the fixed samples were embedded in paraffin and sectional into a thickness of 5 μm and then stained with H&E. The stained sections of each test sample then were examined using light microscopy (Nikon Microphoto-FXA) for tissue inflammatory reaction and tissue regeneration. The number of inflammatory cells observed in each studied case was quantified with a computer-based image analysis system (Image-Pro® Plus, Media Cybernetics, Silver Spring, MD, USA) at a 200× magnification (28). Also, the density of neocapillaries (angiogenesis) and their depth infiltrated (in percentage of the depth of the whole test sample) into each studied sample were quantified with the same image analysis system (28). A minimum of five fields was counted for each retrieved sample. Additional sections were stained with Masson's trichrome to visualize collagen (blue staining).

Immunohistochemical staining was performed on the paraffin sections with a labeled streptavidin-biotin immunoenzymatic antigen detection system (DAKO LSAB 2 System, Dako Co., Carpinteria, CA, USA) (29). The paraffin sections were digested enzymatically with pepsin (1 mg/ml in 0.01 N HCl) for 30 min at 37°**C**. Collagen type I and III mouse monoclonal antibodies were obtained from ICN Biomedicals Inc. (Aurora, OH, USA). The subcutaneous tissue of a healthy rat and the ECM used in the study were used as the positive and negative controls, respectively. Additional sections were stained for factor VIII with immunohistological

technique with a monoclonal anti-factor VIII antibody (Dako Co.) (30).

The extent of vascularization in each retrieved sample was determined by measuring the amount of tissue hemoglobin (31). Test samples were fragmented with a scalpel and immersed in 17 mM Tris-HCl buffer solution (pH 7.6) containing 0.75% 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).

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 ± SD.

RESULTS

Effects of Ginsenoside Re on HUVEC Proliferation

The results of effects of bFGF or ginsenoside Re in varying concentrations on HUVEC proliferation are shown in Fig. 2A. The proliferation activity of each test drug on HUVECs was expressed as the percentage of the sample ab-

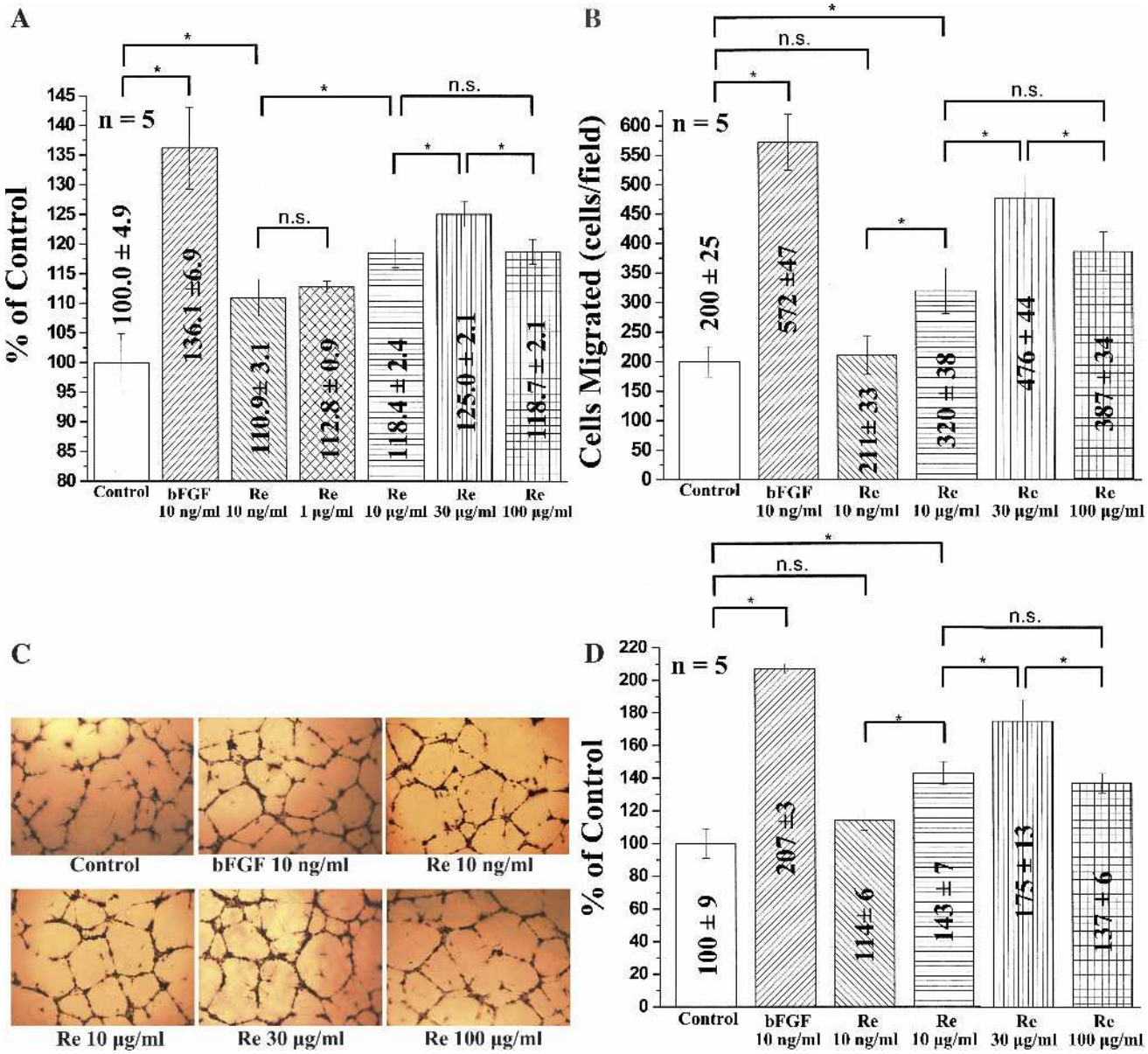

Fig. 2. Effects of ginsenoside Re (Re) on human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation, using bFGF as a control: (A) effects of bFGF or ginsenoside Re on HUVEC proliferation; (B) effects of bFGF or ginsenoside Re on HUVEC migration obtained in a Transwell-plate assay; (C) photomicrographs showing effects of bFGF or ginsenoside Re on tube formation by HUVECs (40× magnification); (D) quantification of tube formation in the presence or absence of bFGF or ginsenoside Re. 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; *statistical significance at a level of $p < 0.05$. Data expressed as mean ± SD.

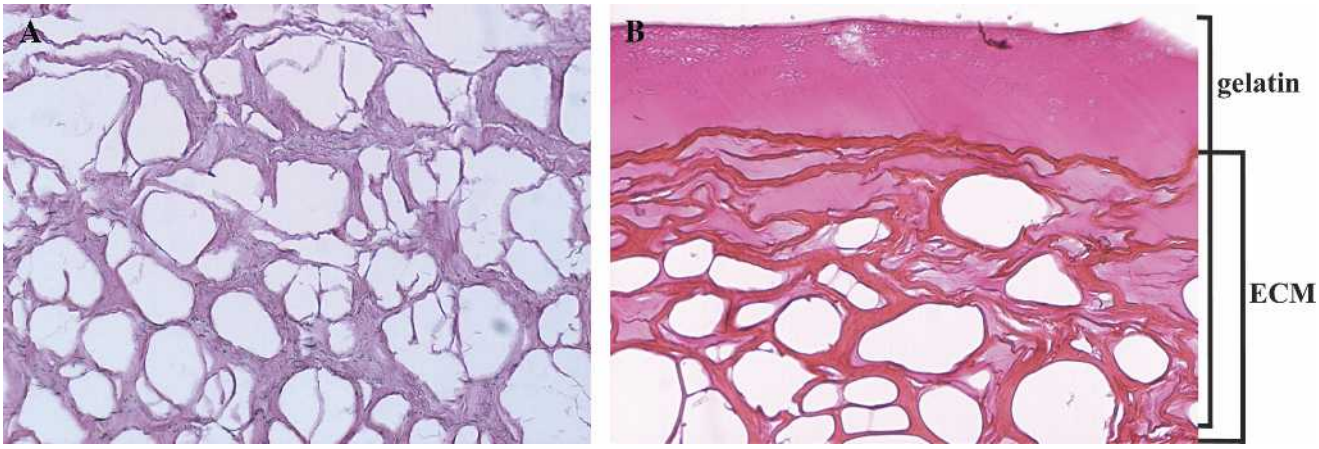

Fig. 3. (A) Photomicrograph of the porous acellular bovine pericardium fixed with genipin prepared in the study as an extracellular matrix (ECM) stained with H&E; (B) photomicrograph of the ECM dip-coated with a gelatin hydrogel incorporated with ginsenoside Re stained with H&E before implantation (200× magnification).

sorbance to that of untreated control cells estimated by the MTS assay. As shown, for bFGF (10 ng/ml), HUVEC proliferation was increased 36% of untreated control cells ($p < 0.05$). At 10 ng/ml ginsenoside Re, cell proliferation was increased 11% of untreated cells, which was found to be significant ($p < 0.05$). The effect of ginsenoside Re on HUVEC proliferation was found to be dose-dependent and had a maximal level at 30 μg/ml ginsenoside Re, an increase of 25% of untreated control cells ($p < 0.05$).

Effects of Ginsenoside Re on HUVEC Migration

HUVECs treated with bFGF showed approximately more than three times the migratory activity of untreated control cells (Fig. 2B, $p < 0.05$). It was observed that the effect of ginsenoside Re on HUVEC migration was dose-dependent. Maximal migration was achieved at 30 μg/ml ginsenoside Re (nearly more than 2.5 times of untreated cells, $p < 0.05$) and migration diminished at a higher concentration (100 μg/ml, $p < 0.05$).

Effects of Ginsenoside Re on HUVEC Tube Formation

As shown in Fig. 2C, after HUVECs were seeded on a Matrigel substrate, they attached, migrated, and formed tubular structures. Twelve hours after seeding, HUVECs formed networks of capillary-like tubes after stimulation with 10 ng/ml of the angiogenic factor bFGF. In contrast, HUVECs formed incomplete and narrow tube-like structures in the absence of angiogenic factor (the control group). Networks similar to that observed with bFGF were formed in the presence of 30 μg/ml ginsenoside Re. 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 control. Figure 2D shows that 10–100 μg/ml of ginsenoside Re stimulated HUVEC tube formation in a dose-dependent manner, and this stimulation was statistically significant as compared to the control group ($p < 0.05$). The maximal activity was shown at 30 μg/ml ginsenoside Re (approximately 1.8-fold of untreated control cells, $p < 0.05$), which was slightly less than that of 10 ng/ml bFGF (about 2-fold of untreated control cells, $p < 0.05$).

Properties of the Prepared ECMs

As shown in Fig. 3A, the prepared ECM revealed large open spaces (pores). The pore size and porosity of the prepared ECM were approximately 159.8 ± 26.7 μm and 94.9 ± 1.7%, respectively, while its denaturation temperature and fixation index measured were 74.5 ± 0.3°C and 57.8 ± 5.4%, respectively ($n = 5$). Angiogenesis and tissue regeneration in the prepared ECM loaded with ginsenoside Re at 70 μg (ECM/Re, Fig. 3B) were investigated subcutaneously in a rat model. The ECM dip-coated in the drug-free gelatin solution (ECM/control) and loaded with 0.7 μg bFGF (ECM/bFGF) were used as blank and positive controls, respectively.

Gross Examinations

Figure 4 presents photographs of each studied group retrieved at 1 week postoperatively. As shown, after genipin

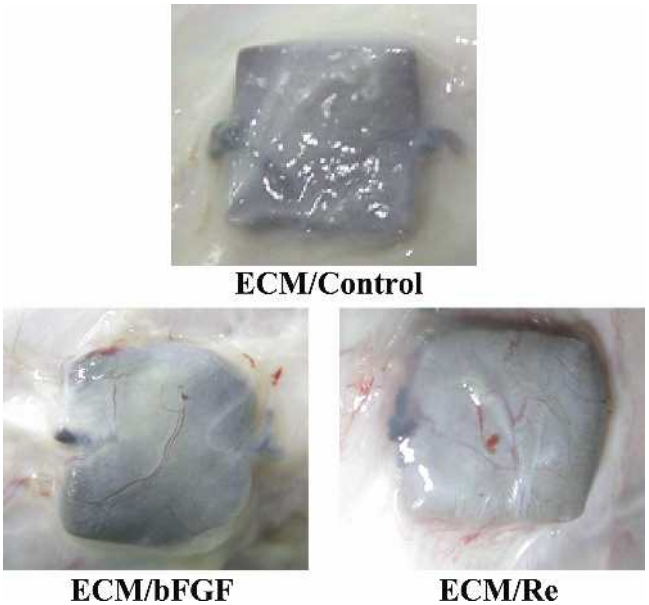

Fig. 4. Photographs of the ECM dip-coated in the drug-free gelatin solution (ECM/control), the ECM incorporated with bFGF (ECM/bFGF), and the ECM incorporated with ginsenoside Re (ECM/Re) retrieved at 1 week postoperatively.

fixation, the color of test ECMs became dark-bluish. A thin layer of transparent tissue enriched with blood capillaries was observed on the surfaces of the ECM/bFGF and ECM/Re. In contrast, there was no macroscopic evidence of any angiogenesis on the surface of the ECM/control.

Histologic Findings

Figure 5A–5C shows photomicrographs of the ECM/control, ECM/bFGF, and ECM/Re stained with H&E retrieved at 1 week postoperatively. The solid lines in the photomicrographs represent the interface between the host tissues (rat) and the implanted test ECMs. Figure 5D presents a photomicrograph of the ECM/Re stained with a monoclonal antibody against factor VIII. As shown, inflammatory cells and neocapillaries were able to infiltrate into the open spaces of all test ECMs. In-growing capillaries (with red blood cells) were coated with an inner endothelial layer (Fig. 5D). The amount of inflammatory cells infiltrated into the ECM/control was the most remarkable among all studied groups ($p < 0.05$, Fig. 6A). The density of neocapillaries (Fig. 6B) and their depth infiltrated (Fig. 6C) into the ECMs loaded with bFGF (the ECM/bFGF) or ginsenoside Re (the ECM/Re) were significantly greater than the control ECM without drug loading (the ECM/control, $p < 0.05$). Additionally, the tissue hemoglobin contents observed in the ECM/bFGF and ECM/Re were statistically larger than the ECM/control (Fig. 6D, $p < 0.05$).

Figures 7A, 7C, and 7E presents photomicrographs of each studied group retrieved at 1 month postoperatively stained with H&E. As shown, the inflammatory cells in the ECM/Re had almost disappeared (Fig. 7E), while there were still some inflammatory cells observed in the ECM/bFGF (Fig. 7C). Additionally, neocapillaries together with neo-connective-tissue fibrils and fibroblasts (migration from the host tissue) were found to fill the pores in the ECM/bFGF and ECM/Re, indicating that the tissue was being regenerated (Figs. 7C and 7E). In contrast, there were still a large number of inflammatory cells with minimal neocapillaries and neo-connective-tissue fibrils seen in the ECM/control (Fig. 7A). The density of neocapillaries and the content of tissue hemoglobin observed in the ECM/Re were significantly greater than their counterparts observed at 1 week postoperatively (Figs. 6B and 6D, $p < 0.05$), whereas those seen in the ECM/bFGF stayed approximately the same ($p > 0.05$).

The neo-connective-tissue fibrils observed in the ECM/bFGF and ECM/Re at 1 month postoperatively were identified to be neocollagen regenerated from the host (rat) by Masson's trichrome (Figs. 7D and 7F). The neocollagen regenerated from the host rat contained collagen type I and type III fibrils recognized by the immunochemical staining (Figs. 7G and 7H). The neocollagen fibrils regenerated from the host in these ECMs can be further confirmed by our denaturation-temperature measurements. As shown in Fig. 8, there were two denaturation-temperature peaks observed for the ECM/bFGF and ECM/Re (~59°C and ~73°C). One was the denaturation temperature of the original bovine collagen fixed with genipin (~73°C), and the other was that of the neocollagen fibrils regenerated from the host (i.e., the rat tissue, ~59°C). On the other hand, there was only one denaturation-

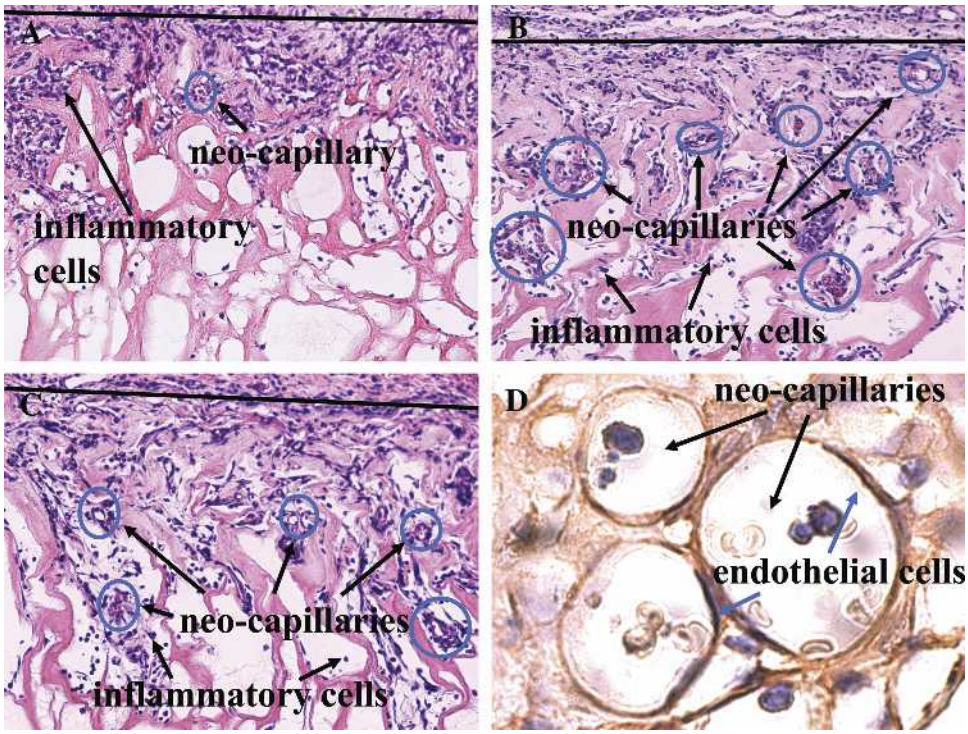

Fig. 5. Histologic evaluation of the tissue responses to test ECMs implanted subcutaneously in a rat model retrieved at 1 week postoperatively: (A) the ECM dip-coated in the drug-free gelatin solution (ECM/control), (B) the ECM loaded with bFGF (ECM/bFGF), and (C) the ECM loaded with ginsenoside Re (ECM/Re) retrieved at 1 week postoperatively stained with H&E (200× magnification); (D) photomicrograph of the ECM/Re retrieved at 1 week postoperatively stained with a monoclonal antibody against factor VIII (800× magnification).

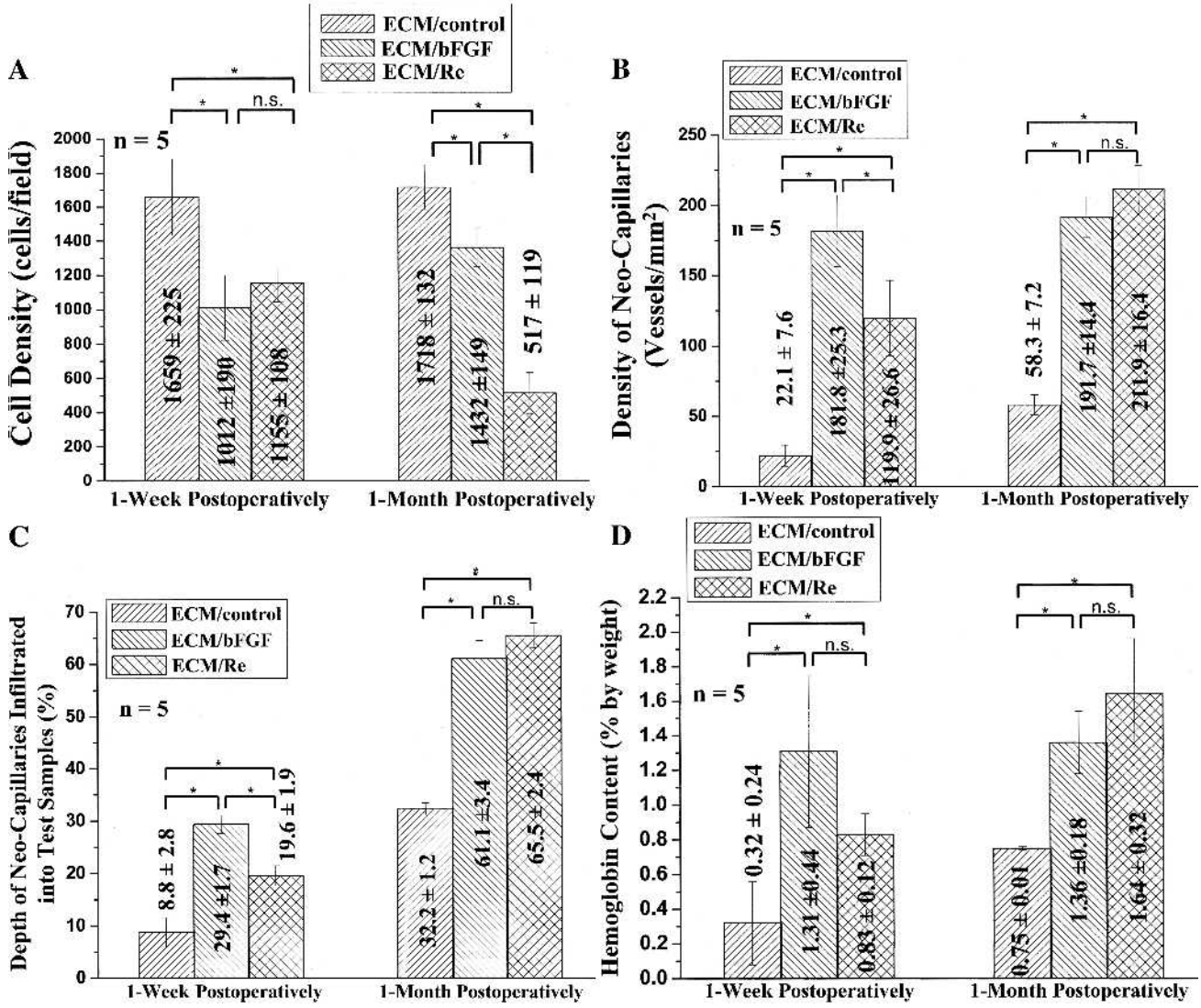

Fig. 6. Quantitative analyses of the cell density and the density of neocapillaries and their depth infiltrated and the tissue hemoglobin content observed in each test ECM retrieved at 1 week and 1 month postoperatively: (A) the cell density observed in each test ECM; (B) the density of neocapillaries and (C) their depth infiltrated into each test ECM (in percentage of the depth of the whole test sample); (D) the tissue hemoglobin content observed in each test ECM. n.s. indicates no statistical difference; *statistical significance at a level of $p < 0.05$. Data expressed as mean ± SD.

temperature peak observed for the ECM/control (the genipin-fixed bovine collagen, ~74°C) due to its minimal regeneration of collagen fibrils from the host at this time.

DISCUSSION

In the study, effects of ginsenoside Re on HUVEC behaviors *in vitro* and on angiogenesis and tissue regeneration in a genipin-fixed porous acellular bovine pericardium (ECM) *in vivo* were investigated, using bFGF as a control. bFGF is one of the endogenous angiogenic factors that stimulates proliferation and migration of endothelial cells (32) and was reported to have various biological activities available to tissue regeneration and tissue engineering (7,33). The *in vitro* models used in the study were similar to those reported in the literature and are appropriate models to examine various aspects of angiogenic behaviors of ginsenoside Re (16). After disruption of the vessel wall, endothelial cells must adhere to matrices in the stromal space, then proliferate, migrate out from the original vascular structure, and organize into new vascular structures (20). Each of these activities is modeled in part by one of our *in vitro* assays.

The sensitivity of HUVECs to ginsenoside Re was evaluated by the enhancement of cell growth after 48 h incubation with varying concentrations of ginsenoside Re. The number of viable cells was estimated by the MTS method using CellTiter 96 Aq_{ueous} One Solution Reagent. The absorbance of the formazan product in each well was read at 490 nm, which was directly proportional to the number of living cells or the activity of cells in culture (17,18). As shown in Fig. 2A, in the presence of bFGF or ginsenoside Re, HUVEC proliferation was increased significantly. The ability of ginsenoside Re to enhance HUVEC migration was tested in Transwell plates. It was found that both bFGF and ginsenoside Re promoted HUVEC migration (Fig. 2B).

The enhancement of HUVEC migration by ginsenoside Re suggested that this agent might also enhance HUVEC tube formation *in vitro*. It was observed that bFGF and gin-

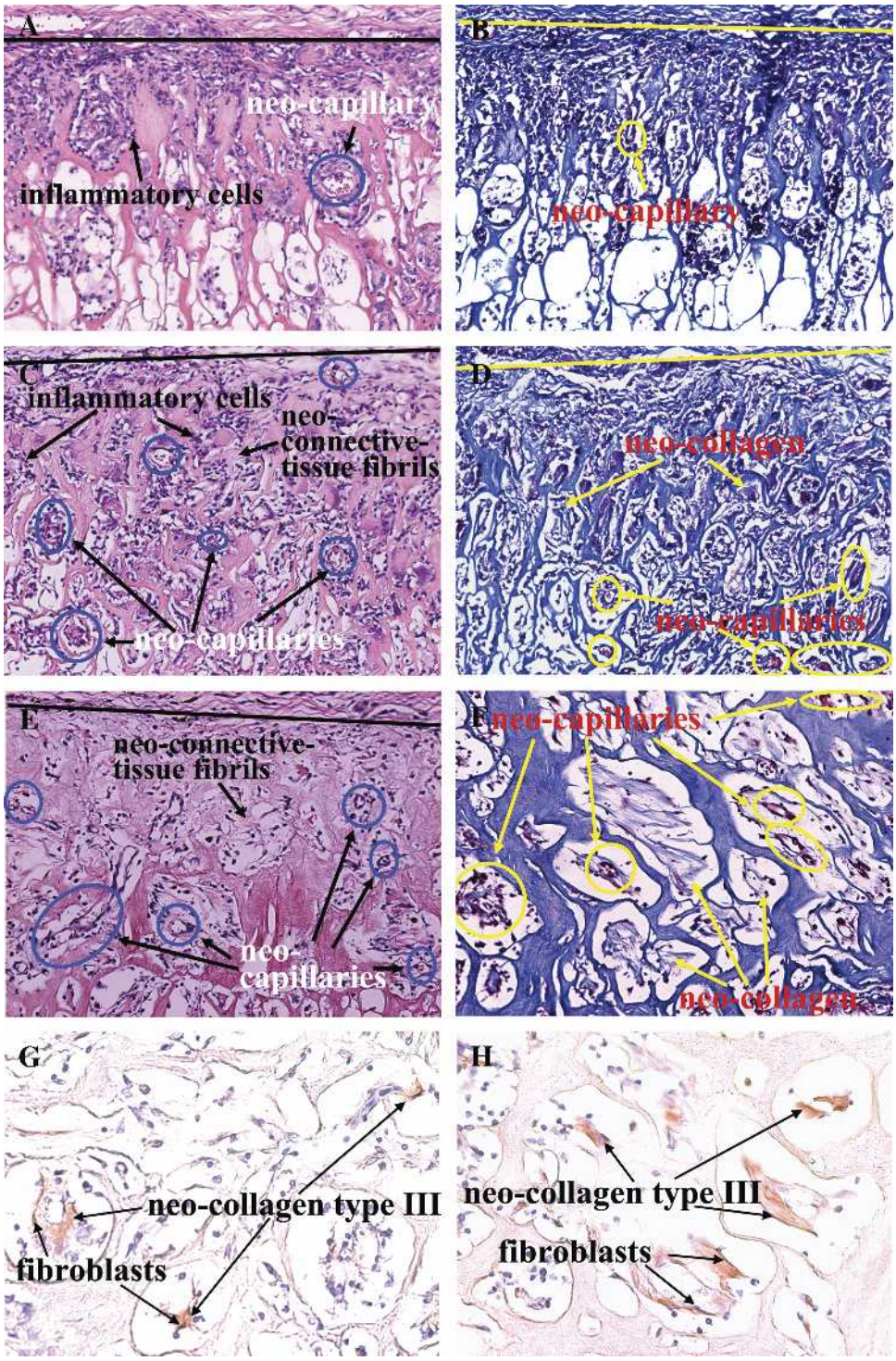

Fig. 7. Histologic evaluation of the tissue responses to test ECMs implanted subcutaneously in a rat model retrieved at 1 month postoperatively: photomicrographs of the ECM/control stained (A) with H&E and (B) with Masson's trichrome, the ECM/bFGF stained (C) with H&E and (D) with Masson's trichrome, and the ECM/Re stained (E) with H&E and (F) with Masson's trichrome (200× magnification); photomicrographs of (G) the ECM/bFGF and (H) the ECM/Re obtained by the immunohistochemical staining to identify neocollagen type III (400× magnification).

senoside Re significantly increased formation of capillary-like network by HUVECs on Matrigel (Figs. 2C and 2D). During angiogenesis, activated existing endothelial cells proliferate, and their mobile activity is increased. The mobile endothelial cells migrate toward the attractants and connect each other to form tube-like structures *in vitro* (Fig. 2C) or neocapillaries *in vivo* (Fig. 4 and Fig. 5D). The ability of endothelial cells to form a network of tubular structures across the surface of a

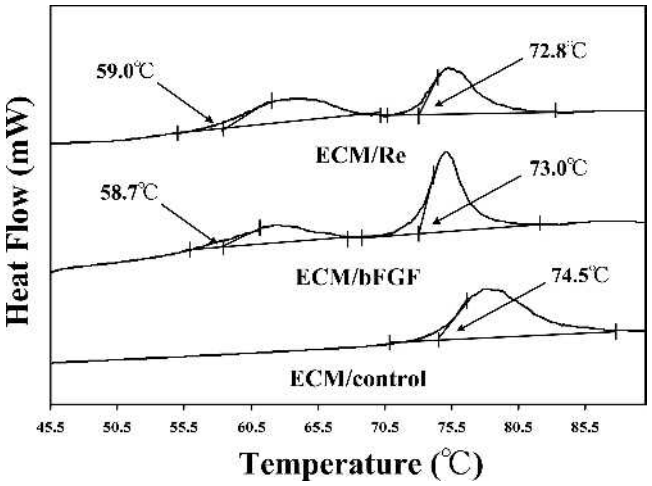

Fig. 8. Thermograms of the ECM dip-coated in the drug-free gelatin solution (ECM/control), the ECM incorporated with bFGF (ECM/bFGF), and the ECM incorporated with ginsenoside Re (ECM/Re) retrieved at 1 month postoperatively measured by a differential scanning calorimeter.

Matrigel substratum is a complex phenomenon that combines elements of attachment, migration, organization, and differentiation (34). Other cell types such as salivary gland, mammary, renal tubular, or bone cells also exhibit organization on Matrigel (35–37). The complex organizational behavior of HUVECs on Matrigel models the type of coordinated activities required for angiogenesis by endothelial cells (20). Thus, although the *in vitro* Matrigel model does not represent true angiogenesis by itself, it suggested that ginsenoside Re is important for many of activities that contribute to vessel formation.

Additionally, it was found that ginsenoside Re possesses dose-dependent properties on proliferation, migration, and tube formation assays of HUVECs (Figs. 2A, 2B, and 2D). Proliferation, migration, and tube formation were maximal at 30 μg/ml ginsenoside Re, with responses decreasing somewhat at higher doses. This phenomenon may be due to the effect of cytotoxicity of ginsenoside Re when the concentration was increased to 100 μg/ml. Thus, ginsenoside Re enhances multiple components of angiogenic activity *in vitro* in a dose-dependent manner. For bFGF, such dose-dependent pattern was also reported in the literature (38). It was recently reported that another component of saponin isolated from *Panax ginseng*, ginsenoside Rg1, also had similar effects on HUVECs (39).

In the *in vivo* study, the acellular tissue was further treated with acetic acid and a collagenase solution to increase its pore size, porosity, and pore interconnectivity. It is generally accepted that a tissue-engineering extracellular matrix must be highly porous for a sufficient cell density to be seeded *in vitro*, for blood invasion to occur *in vivo*, and for oxygen and nutrients to be supplied to cells (40). In our previous study, it was found that optimization of the porous structures of acellular tissues can significantly increase the rate of angiogenesis (in-growth of microvessels) (24). However, in tissue repair or regeneration, this natural angiogenic response is insufficient to maintain large cell masses required to provide therapeutic support (4). Therefore, the feasibility of incorporating ginsenoside Re in a porous acellular bovine pericardium for angiogenesis and tissue regeneration was evaluated subcutaneously in a rat model.

The prepared porous acellular tissues were fixed by genipin, a naturally occurring cross-linking agent isolated from the fruits of *Gardenia jasminoides* ELLIS (41,42). Genipin and its related iridoid glucosides have been widely used as an antiphlogistic and cholagogue in herbal medicine (43). It was found in our previous study that genipin can react with free amino groups such as lysine, hydroxylysine, or arginine residues in biological tissues (44). The reaction mechanism of genipin with biological tissues was previously proposed (45).

The *in vivo* results obtained at 1 week postoperatively showed that the density of neocapillaries (Fig. 6B) and the content of tissue hemoglobin (Fig. 6D) in the ECMs were significantly enhanced by bFGF or ginsenoside Re. These results indicated that angiogenesis in the ECMs was significantly enhanced by loading with bFGF or ginsenoside Re. It is known that site-specific release of angiogenic molecules may provide an efficient means of stimulating localized vessel formation (6). The ECMs dip-coated with a gelatin hydrogel incorporated with bFGF or ginsenoside Re prepared in the study (Fig. 3B) may allow one to optimize this process by varying the concentrations of gelatin used or cross-linked with a cross-linking agent.

At 1 month postoperatively, vascularzied neo-connective-tissues fibrils were found to fill the pores within the ECMs (Figs. 7A–7H). It was observed that there were significantly more neocapillaries (Fig. 6B) and tissue hemoglobin (Fig. 6D) measured in the ECM/bFGF and ECM/Re than in the ECM/control. These results indicated that in the same way as bFGF, ginsenoside Re-associated induction of angiogenesis enhanced tissue regeneration, supporting the concept of therapeutic angiogenesis in tissue-engineering strategies.

There were significant increases in the density of neocapillaries (Fig. 6B) and the hemoglobin content (Fig. 6D) for the ECM loaded with ginsenoside Re from 1 week to 1 month postoperatively. These results suggested that the released ginsenoside Re continued to be effective in enhancing angiogenesis. In contrast, although bFGF can enhance angiogenesis at 1 week postoperatively, it is difficult to achieve long-term delivery of functional properties because of the limitations of protein stability. It was reported that at physiologic pH and temperature, the *in vitro* half-life of bFGF activity is approximately 12 h (46,47).

CONCLUSIONS

In conclusion, the *in vitro* results showed that proliferation, migration, and tube formation of HUVECs were all enhanced in the presence of ginsenoside Re in a dose-dependent manner. Additionally, the *in vivo* results demonstrated that angiogenesis and tissue regeneration were enhanced by ginsenoside Re loaded in the ECM. These results suggested that ginsenoside Re is a novel angiogenic agent and can be loaded in an ECM for accelerating tissue regeneration. This may open up the possibility of using nonpeptide molecule–based therapeutic approaches in the management of angiogenesis for tissue regeneration.

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