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Effects of ginseng saponins isolated from Red Ginseng roots on burn wound healing in mice

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1 We recently demonstrated that ginsenoside Rb_1 ($C_{54}H_{92}O_{23}$, molecular weight 1108) isolated from ginseng, when intravenously infused into rats with permanent middle cerebral artery occlusion, reduced cerebral infarct volume and ameliorated place navigation disability of the animals, through an anti-apoptotic action and possibly promotion of vascular regeneration. To investigate the ginsenoside Rb_1-mediated vascular regeneration *in vivo* in a more easily accessible experimental systems, we made a burn wound on the backs of mice and topically applied either Vaseline (vehicle) alone or Vaseline containing low doses of ginsenoside Rb_1 to the wound.

2 Surprisingly, we found that ginsenoside Rb_1 at low concentrations ($100\,pg\,g^{-1}$, $1\,pg\,g^{-1}$ and $10\,fg\,g^{-1}$ ointment) exhibited the strongest burn wound-healing action. Furthermore, ginsenoside Rb_1 (100 fg–1 ng per wound) increased neovascularization in the surrounding tissue and production of vascular endothelial growth factor (VEGF) and interleukin (IL)-1β from the burn wound, compared to those mice with burn wounds treated with vehicle alone.

3 In human keratinocyte cultures (HaCaT cells), ginsenoside Rb_1 ($100\,fg\,ml^{-1}$ to $1\,ng\,ml^{-1}$) enhanced VEGF production induced by IL-1β and expression of hypoxia-inducible factor (HIF)-1α.

4 These findings suggest that the promotion of burn wound healing by ginsenoside Rb_1 might be due to the promotion of angiogenesis during skin wound repair *via* the stimulation of VEGF production, through the increase of HIF-1α expression in keratinocytes, and due to the elevation of IL-1β resulting from the macrophage accumulation in the burn wound.
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Abbreviations: DMEM, Dulbcecco's modified Eagle's medium; HIF-1α, hypoxia-inducible factor-1α; IL, interleukin; VEGF, vascular endothelial growth factor

Introduction

Red Ginseng root (*Panax ginseng* CA Meyer) is used clinically in China, Korea and Japan for various diseases, including atherosclerosis, liver dysfunction, cerebrovascular diseases, hypertension and post-menopausal disorder (Yamamoto, 1988). Red Ginseng root extracts have also been used clinically as topical treatments for atopic suppurative dermatis, wounds and skin inflammation. The physiological action of ginseng, and in particular of the beneficial effects of Red Ginseng root extract on the skin, however, is not yet well understood. It has been reported that ginsenoside Rg_1 promoted functional neovascularization into a polymer scaffold *in vitro* and angiogenesis *in vivo* (Sengupta *et al*., 2004). On the other hand, ginsenoside Rb_1 and Rb_2 are reported to inhibit angiogenesis, whereas ginsenoside Rb_2 improved wound healing by enhancing epidermal cell proliferation (Sato *et al*., 1994; Choi, 2002; Sengupta *et al*., 2004). Thus, there are perplexing contradictions in the reported effects of various ginsenosides on wound healing.

Therefore, to clarify the differing effects of various ginsenosides, we examined the effects of total ginseng saponins and various ginsenosides on wound healing using a burn-treated mouse model. It is well known that burns initially induce coagulative necrosis and cause scar formation after repair. Macrophages migrate to the injured area to kill invading organisms and produce cytokines that recruit other inflammatory cells that are responsible for the diverse effects of inflammation (O'Riordain *et al*., 1992; Kataranovski *et al*., 1999). Angiogenesis in the injured area is closely associated with the process of wound healing (Atavilla *et al*., 2001). Moreover, growth factors and cytokines are central to the wound-healing process (Brown *et al*., 1992; Martin, 1997; Sen *et al*., 2002). Thus, burn wound healing is a complex process, involving inflammatory aspects such as monocyte migration and cytokine production, and growth factors and angiogenesis during re-epithelialization. In preliminary experiments, we found that the total ginseng saponins isolated from Red Ginseng roots accelerated burn wound healing in mice.

Therefore, we attempted to examine the various ginseng saponins isolated from Red Ginseng roots using the same burn wound model. Among these ginseng saponins, we found that ginsenoside Rb_1 had the strongest burn wound-healing action. In this study, we examined the effects of ginsenoside Rb_1 on vascular endothelial growth factor (VEGF) and Ki-67

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expression, and the accumulation of macrophages in the regenerating skin area of burn wounds, by immunohistochemistry. Furthermore, we attempted to clarify the mechanisms of the effects of ginsenoside Rb_1 on burn wound healing.

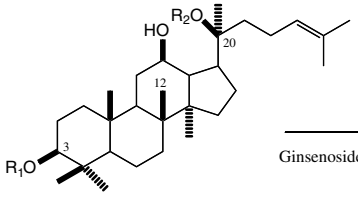
Methods

Preparation of ginsenosides

Ginseng saponins were isolated by the method described by Shibata and co-workers (Nagai *et al*., 1971; Sanada *et al*., 1974a, b; Shibata *et al*., 1985; Shibata, 2001). Briefly, six ginsenosides were isolated and purified from the crude saponin fractions of roots of *Panax ginseng* CA Meyer, Korean Red Ginseng, by repeated column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (65 : 35 : 10, v v^{-1}) and octadecylsilyl silica with MeOH–H_2O (1 : 1 to 7 : 3, v v^{-1}). The purity of each of the six ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1) used in this study was more than 99.99%, as determined by high-performance liquid chromatography. The following ginseng saponins were used in the present investigation: glycosides of protopanaxadiol such as ginsenosides Rb_1, Rb_2, Rc and Rd, and glycosides of protopanaxatriol such as ginsenosides Re and Rg_1 (Figure 1). The various ginsenosides were used in white Vaseline ointment or dissolved in saline (NaCl, 0.9% w v^{-1}) as indicated in the text.

Cells

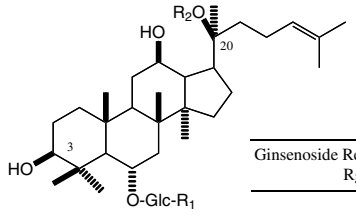
The human keratinocyte cell line (HaCaT cells) was obtained from the Department of Dermatology, School of Medicine, Ehime University and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Human adult dermal microvascular endothelial cells were purchased from Cell Systems (Kirkland, WA, U.S.A.), seeded onto collagen (Type I)-coated six-well culture plates and maintained in Clonetic's microvascular endothelial basal medium supplemented with growth factor.



20(S) Protopanaxadiol-type

	R_1	R_2
Ginsenoside Rb_1:	-Glc^2-Glc	-Glc^6-Glc
Rb_2:	-Glc^2-Glc	-Glc^6-Ara(p)
Rc :	-Glc^2-Glc	-Glc^6-Ara(f)
Rd :	-Glc^2-Glc	-Glc

(p): pyranosyl, (f): furnaosyl



20 (S) Protopanaxatriol-type

	R_1	R_2
Ginsenoside Re:	-Rham	-Glc
Rg_1:	-H	-Glc

Figure 1 The structures of various ginsenosides.

Animals

Male Balb/c mice (5 weeks old) were obtained from Clea Japan (Osaka, Japan), housed for 1 week in a room with controlled temperature at 25 ± 1°C and relative 60% humidity, and given free access to standard laboratory diet and water. Mice were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University, and the experimental protocol was approved by the Animal Studies Committee of Ehime University.

Measurement of burn wound healing

To examine the effects of the total ginseng saponins from Red Ginseng and various isolated ginseng saponins (protopaxadiol and protopanaxatriol types) on the burn wound-healing process, burn wounds were created on the backs of male Balb/c mice under anesthesia with pentobarbital (50 mg kg^{-1}; i.p.). Hair was removed from the backs of mice using hair remover under anesthesia. The backs of the mice were subsequently wiped with 37°C-distilled water and 70% ethanol. A customized soldering iron was used to cause the burn to the skin on the backs of the mice. The custom-made tip of the soldering iron was 7 mm in diameter with a heating ring of 1 mm thickness around the outside of the tip, which reached a temperature of 250°C. After the burn wound was made by applying the soldering iron to the skin for 10 s, a sterile biopsy punch (8 mm diameter, Kai Industries Co., Tokyo, Japan) was used to excise the burnt skin, leaving the underlying fasciae intact. All surgical treatments were performed under anesthesia with pentobarbital. Indicated amounts of total ginseng saponins or the six isolated ginseng saponins (ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1) were applied to the burn wound surface and then covered with film dressing (PERME-AIDS, Nitto Medical Co., Tokyo, Japan) for 19 consecutive days. White Vaseline alone was applied to control mice using the same schedule. The burn wound area was measured every other day by direct measurement with calipers. On day 20, mice were killed by cervical dislocation and the wounded skin was removed for analysis.

Histological examination

All samples of burn wounded skin were fixed in 10% buffered formalin for at least 24 h, progressively dehydrated in solutions containing an increasing percentage of ethanol (70, 80, 95 and 100%), cleared in Histoclear (AS-ONE, Tokyo, Japan), embedded in paraffin under vacuum, sectioned at 5 μm thickness, de-paraffinized and stained with hematoxylin and eosin. After the paraffin-embedded skin tissue sections were de-paraffinized, the expression of Ki-67-positive cells and the accumulation of macrophages in the burn wound area were examined by the immunoperoxidase technique using anti-mouse Ki-67 and anti-mouse macrophage antibodies, respectively.

Measurement of the IL-1β, VEGF and angiogenesis in the burn wound

The backs of mice were subjected to burn wound by the same method as described above. A polyethylene filter pellet (about 8 mm diameter, 3 mm thickness) containing the indicated

amounts of basic fibroblast growth factor (bFGF) or ginsenoside Rb_1 was applied to the burn wound surface, and covered with film dressing. On days 1, 3, 5 and 7, the filter pellets were removed and replaced with fresh filter pellets. On day 9, after the filter pellets were removed, the mice were killed. For control mice, filter pellets containing saline alone were applied according to the same schedule. Immediately after removal, phosphate-buffered saline (PBS, pH 7.0) (200 μl) was added to each filter pellet and mixed for 10 min. The filter pellet was then removed and the extract centrifuged at $1000 \times g$ for 10 min at 4°C. The cell pellets thus obtained were suspended in PBS, and the cell numbers were counted using a cell counter machine. The numbers of macrophage and polymorphonuclear leukocytes (PMNs) in the filter pellet were measured by smear testing. The supernatants from the centrifugation were stored at −80°C until they were analyzed for their content of interleukin (IL)-1β and VEGF, using mouse IL-1β and VEGF ELISA kits. Any angiogenesis in the site surrounding the burn wound was photographed using a stereoscopic microscope (SMZ 800, Nikon, Tokyo, Japan) and the area and length of angiogenesis were measured using a Coordinating Area and Curvimeter Machine (X-PLAN 360 dII, Ushitaka, Tokyo, Japan).

*Measurement of VEGF production and HIF-1α expression in HaCaT cells (*in vitro*)*

Human keratinocytes (HaCaT cells) were seeded (2×10^5 cells) on 10-mm dish culture plates and cultured for 72 h. After reaching 80% confluency, cells were treated with the indicated amounts of ginsenoside Rb_1 in the presence or absence of IL-1β (20 ng ml^{-1}) for 1, 2 or 6 h. VEGF levels in the medium were determined by ELISA, using a human VEGF kit. After removal of medium, nuclear extracts of the cells were prepared according to the methods of Dignam *et al.* (1983) for the measurement of hypoxia-inducible factor (HIF)-1α protein. Briefly, the cells were washed with ice-cold PBS and pelleted. The cells were resuspended in 800 μl of buffer A (10 mM Hepes buffer (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 5 μg ml^{-1} aprotinine, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin A, 1 mM Na_3VO_4 and 10 mM β-glycerophosphate) and allowed to swell for 10 min, and then the cells were pulled 10 times into a 25G three-eighths needle for cell membrane disruption, and the nuclei pelleted in a microcentrifuge. The cell nuclei were resuspended in 50 μl of buffer B (20 mM Hepes buffer (pH 7.9) containing 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin A, 1 mM Na_3VO_4 and 10 mM β-glycerophosphate) and incubated on ice for 30 min. After centrifugation, the supernatant was used for the measurement of HIF-1α protein. Samples (20 μg protein) were boiled for 5 min, subjected to electrophoresis on 7.5% polyacrylamide gel and used for Western blot analysis with specific mouse anti-HIF-1α monoclonal antibody or mouse anti-β-actin monoclonal antibody.

Cell proliferation assay

The HaCaT cell lines were plated at a density of 1×10^3 cells well^{-1} in 96-well microtiter plates and incubated at 37°C in 5% CO_2 for 24 h. To investigate the cell proliferation-related effects and cytotoxicity of ginsenoside Rb_1, HaCaT cells were incubated with the indicated amounts of ginsenoside Rb_1 (100 fg ml^{-1}, 10 pg ml^{-1} or 1 ng ml^{-1}) for 24 h. Cell viability in each well was determined by the MTT assay using a Cell counting kit.

Materials

DMEM and endothelial cell growth medium were purchased from Nissui Pharm. Co. (Tokyo, Japan) and Clonetics (San Diego, CA, U.S.A.), respectively, and used as culture media. Antibiotic and antimycotic solution (100×) containing 10,000 U ml^{-1} of penicillin, 10 mg ml^{-1} of streptomycin and 25 μg ml^{-1} of amphotericin B in 9 mg ml^{-1} NaCl was purchased from Sigma (St Louis, MO, U.S.A.). Fibroblast Spray 250 (bFGF 250 μg) was obtained from Kaken Pharmaceutical Co. (Tokyo, Japan). Multiwell plates (6, 12 or 48 wells) were

Table 1 Effects of total ginseng saponins and various ginsenosides isolated from Red Ginseng roots on burn wound healing in Balb/c mice

	No. of animals	*Initial wound area* (mm^2)	*Maximum wound area* (mm^2) *on day 8*	*Final wound area* (mm^2) *on day 20*
Burn wound-treated mice (control 1) (Exp. 1)	6	109.7±9.63	334.2±28.8	53.9±13.1
+Total ginseng saponins				
(100 pg g^{-1} ointment)	7	93.8±9.36	251.1±25.5	22.2±2.6*
(1 μg g^{-1} ointment)	7	101.2±4.12	254.9±24.9	26.1±2.9*
Burn wound-treated mice (control 2) (Exp. 2)	6	108.8±13.5	254.8±19.5	112.8±24.2
+Ginsenoside Rb_1 (100 pg g^{-1}ointment)	6	86.2±7.04	113.5±47.1*	36.1±12.8*
Burn wound-treated mice (control 3) (Exp. 3)	5	81.9±2.67	198.8±10.5	38.6±3.77
+Ginsenoside Rb_2 (1 ng g^{-1} ointment)	5	82.7±3.69	204.5±8.72	32.9±5.21
+Ginsenoside Rc (1 ng g^{-1}ointment)	5	80.0±5.41	172.9±4.84	14.2±1.94*
+Ginsenoside Rd (1 ng g^{-1}ointment)	5	89.8±5.79	206.4±14.5	33.2±3.07
+Ginsenoside Re (1 ng g^{-1} ointment)	5	81.7±5.97	195.5±8.63	28.5±5.92
+Ginsenoside Rg_1 (1 ng g^{-1} ointment)	5	77.4±4.21	193.4±15.7	33.7±4.12

Values are means±s.e. of 5–7 mice. *Significantly different from the control value, $P<0.05$.

purchased from Corning Glass Works (Corning, NY, U.S.A.). Collagen (Type I)-coated six-well plates were purchased from Sumitomo Bakelite (Tokyo, Japan). Human and mouse VEGF ELISA kits and mouse IL-1β were obtained from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Rat monoclonal anti-mouse Ki-67 antibody and rabbit polyclonal anti-rat biotin-labeled immunoglobulin antibody peroxidase-labeled streptavidin were purchased from DakoCytomation (Kyoto, Japan). Mouse monoclonal anti-macrophage antibody (Clone MAC 387) and mouse monoclonal anti-HIF-1α antibody (clone H1α 67) were purchased from Neo Markers (Fremont, CA, U.S.A.) and Novus Biologicals (Littleton, CO, U.S.A.), respectively. The cell counting kit was purchased from Wako Pure Chemical Co. (Osaka, Japan). Other chemicals were of reagent grade.

Statistical analysis

All values are expressed as means ± s.e. Data were analyzed by one-way ANOVA, and then differences among means were analyzed using Fisher's protected least significant difference multiple-comparison test. Differences were considered significant at $P<0.05$.

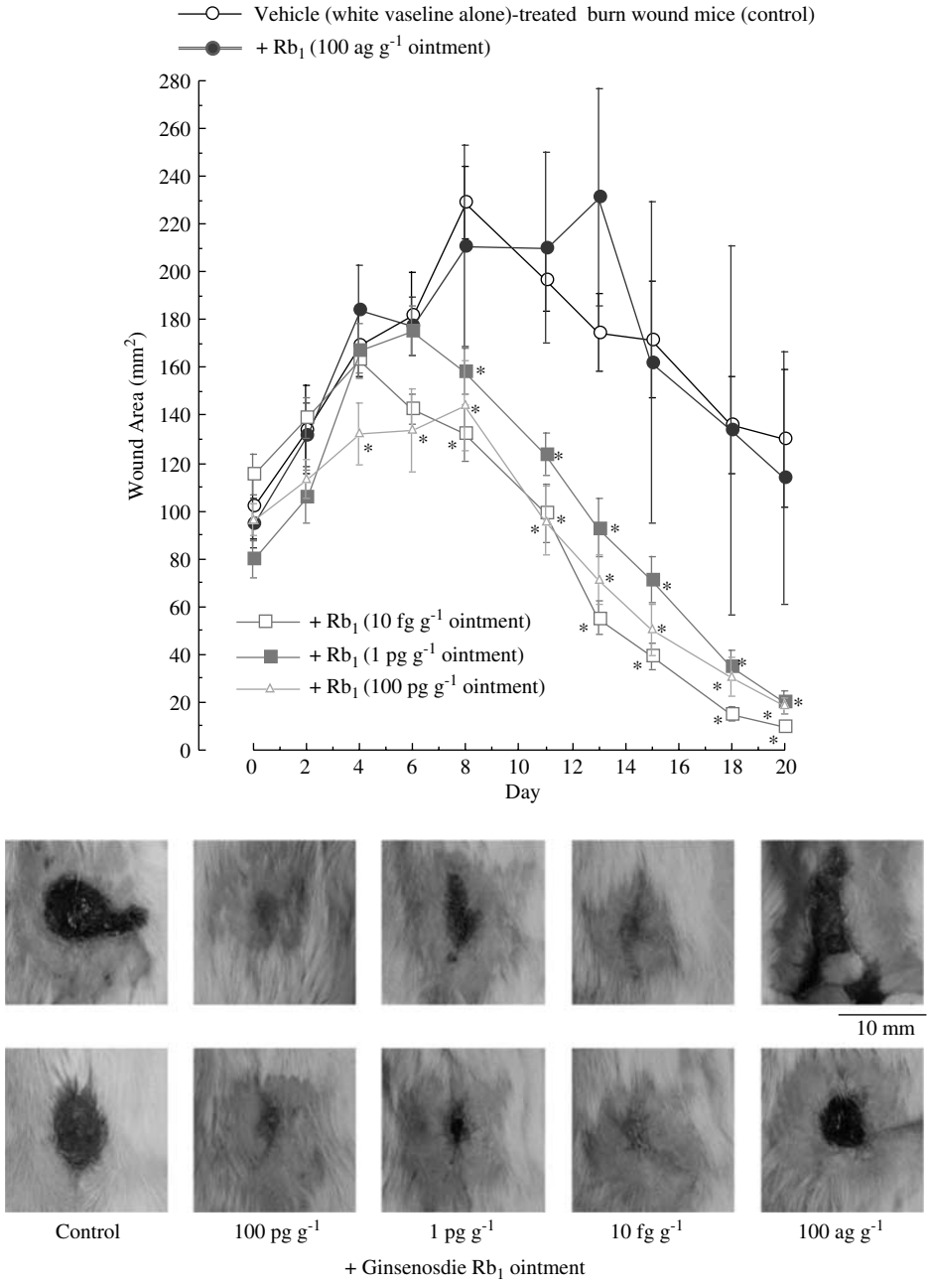


Figure 2 Effects of ginsenoside Rb_1 on burn wound healing in Balb/c mice. Values are means ± s.e. of 6–12 mice. *Significantly different from corresponding values in control group, $P<0.05$. The control group (treated with Vaseline only) and Rb_1 (100 pg g^{-1} ointment)-treated groups consisted of 12 mice each, and the Rb_1 (1 pg g^{-1}, 10 fg g^{-1} and 100 ag g^{-1} ointment)-treated groups consisted of six mice each.

Results

Effects of total ginseng saponins isolated from Red Ginseng on burn wound healing in mice

The area of the wound injury created by heating with the soldering iron at 250°C for 10 s reached a maximum on day 8 (Table 1) and then the wounds were observed to undergo repair. The wound area after day 20 was reduced by treatment with ointment containing total ginseng saponins at concentrations of 100 pg g^{-1} and 1 μg g^{-1}, compared to the wound area of mice treated with white Vaseline alone.

Effects of ginsenoside Rb_1 on burn wound healing

Among the six ginsenosides tested (ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1), ginsenoside Rb_1 enhanced wound healing most strongly (Table 1). Therefore, we examined the effects of lower doses (100 pg g^{-1}, 1 pg g^{-1}, 10 fg g^{-1} and 100 ag g^{-1} ointment) of ginsenoside Rb_1 (Figure 2). The burn wound area in mice treated with ginsenoside Rb_1 in the range of 100 pg g^{-1} to 10 fg g^{-1} was significantly reduced on days 8–20 compared to that in untreated mice, but the ointment with ginsenoside Rb_1 at a dose of 100 ag g^{-1} had no effect.

Effects of ginsenoside Rb_1 on the numbers of infiltrating leukocytes (PMNs and macrophages) and angiogenesis in the burn wound area, and IL-1β and VEGF content in the exudates of burn wounds

The number of macrophages migrating to the burn wound area was increased 1, 3 and 5 days after the application of ginsenoside Rb_1 at a dose of 100 fg, 10 pg or 1 ng per filter pellet compared with that of the vehicle (saline)-treated group (control). Basic FGF (2.5 μg per filter pellet) also increased the migration of macrophages 3, 5, 7 and 9 days after the burn treatment (Figure 3a). On the other hand, the number of PMNs was reduced by ginsenoside Rb_1 (1 ng per filter pellet) 3 days after the burn, but the lower doses of 100 fg and 10 pg, of either ginsenoside Rb_1 or bFGF, had no effect (Figure 3b).

Next, we examined the levels of IL-1β and VEGF in exudates of the burn wounds. The level of IL-1β production in the exudates of the wound area was increased time-dependently over 9 days. Ginsenoside Rb_1 (1 ng per filter pellet) significantly increased the levels of IL-1β on days 1, 3 and 5 compared to the IL-1β levels in vehicle-treated mice (control group), whereas on day 9 the IL-1β level was decreased by the application of ginsenoside Rb_1 (1 ng), as compared to the control group (Figure 4a). IL-1β in the exudates from the wound area was also increased on day 3 by the application of bFGF (2.5 μg per filter pellet) compared to control mice (Figure 4a). The VEGF level in the exudates from the wound area increased until day 5, and then decreased. The application of ginsenoside Rb_1 increased VEGF levels on days 1 and 9 compared to control mice (Figure 4b). On the other hand, the application of bFGF did not affect the VEGF level on day 1 (Figure 4b). There were no significant differences in protein levels in the exudates of the burn wound among untreated and ginsenoside Rb_1- or bFGF-treated mice (data not shown).

Based on these findings, we examined the effects of ginsenoside Rb_1 on neovascularization in the tissue surrounding the wound area. As shown in Figure 5 and Table 2, neovascularization from the tissue surrounding the wound area was expressed as blood vessel length (mm per field) and as

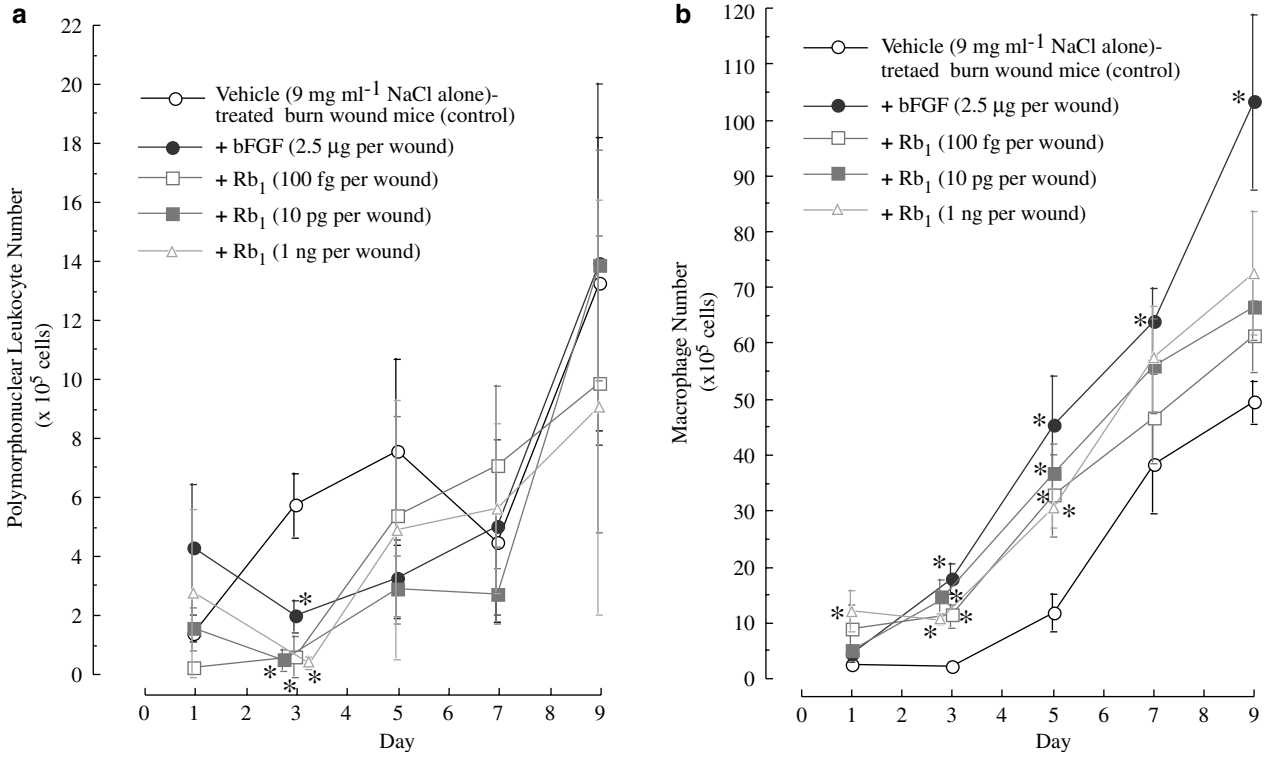


Figure 3 Effects of ginsenoside Rb_1 and bFGF on PMN (a) and macrophage (b) migration to the burn wound area in mice. Values are means ± s.e. of six mice. *Significantly different from control values, $P<0.05$.

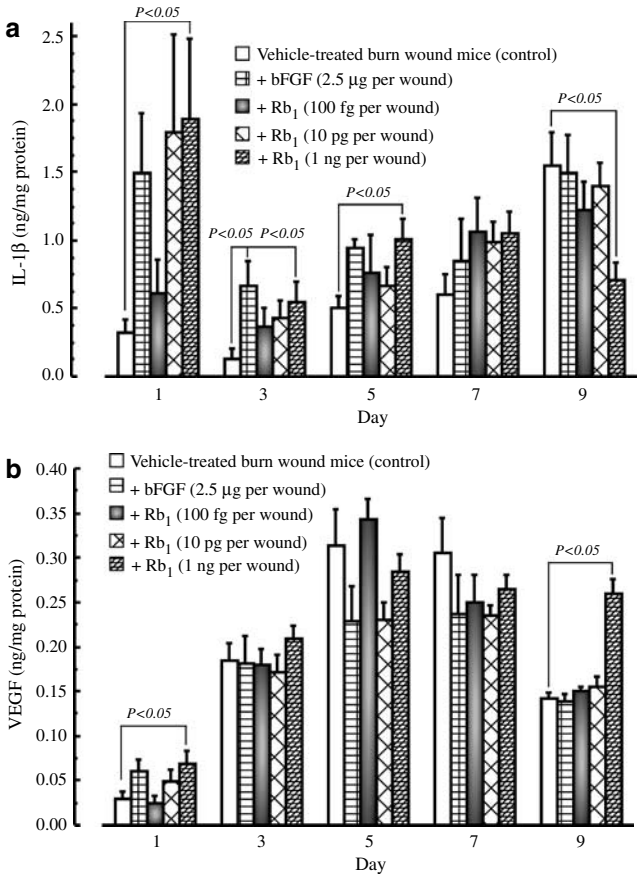


Figure 4 Effects of ginsenoside Rb_1 and bFGF on IL-1β (a) and VEGF (b) content in the exudates of the burn wound area in mice. Values are means$\pm$s.e. of six mice. *Significantly different from control values, $P<0.05$.

blood vessel area (mm^2 per field). The application of bFGF (2.5 μg per filter pellet) or ginsenoside Rb_1 (100 fg, 10 pg and 1 ng per filter pellet) for 9 days increased the blood vessel length by about 3- to 3.5-fold and the corresponding area by about 3.5- to 5.0-fold, compared to control mice (Figure 5 and Table 2).

Immunohistochemical analysis of the effect of ginsenoside Rb_1 treatment on wound healing in mice

As shown in Figure 2, we observed enhanced healing of burn wounds treated with ginsenoside Rb_1. After 20 days of repair, ginsenoside Rb_1-treated wounds appeared to be markedly reduced in diameter and almost completely re-epithelialized as compared to vehicle-treated wounds (control). Therefore, on day 9, we examined the effects of ginsenoside Rb_1 histologically in burn wounds. Hematoxylin–eosin -stained sections of vehicle- or ginsenoside Rb_1-treated wounds consistently showed histological differences (Figure 6a). After 8 days of treatment with ginsenoside Rb_1 (100 fg, 10 pg and 1 ng per filter pellet), burn wounds were characterized by a dense neoepithelium and neovascularization from the tissue surrounding the wound area (Figures 5 and 6a). As shown in Figure 6a, the epithelium proliferated more in response to the application of ginsenoside Rb_1 (100 fg, 10 pg or 1 ng per filter pellet), as compared to vehicle-treated mice.

The nuclear protein Ki-67 is a well-known marker of cellular proliferation (see Urruticoechea *et al*, 2005). Keratinocytes of hyperproliferative epithelia at the wound margins in vehicle-treated mice were characterized by a diffuse distribution of Ki-67-positive cells. On the other hand, wounds from ginsenoside Rb_1-treated mice showed an increase of Ki-67-positive cells at the epidermal basal layer. The Ki-67-positive cells reached deeper into the granulation tissues after the application of ginsenoside Rb_1, compared to the wounds of vehicle-treated mice (Figure 6b and Table 3). We had found that the migration of macrophages into the wound exudates (Figure 3a) was increased by the application of ginsenoside Rb_1 at 100 fg, 10 pg and 1 ng per filter pellet. Therefore, to clarify whether there were any changes in the number of macrophages in the wound tissues of mice that received ginsenoside Rb_1, we looked for these cells in the histological sections. As shown in Figure 6c, macrophages accumulated in subdermal tissue immediately adjacent to the dermis on 9 days after the burn injury in response to the application of ginsenoside Rb_1 (100 fg, 10 pg or 1 ng per filter pellet).

*Effects of ginsenoside Rb_1 on VEGF production and HIF-1α protein expression with or without IL-1β in cultured HaCaT cells (*in vitro*)*

Although ginsenoside Rb_1 (100 fg ml^{-1}, 10 pg ml^{-1} or 1 ng ml^{-1}) had no effect on the VEGF production in HaCaT cells in the absence of IL-1β, all three concentrations of ginsenoside Rb_1 significantly increased the VEGF production in HaCaT cells exposed to IL-1β for 1 or 2 h (Figure 7a). HIF-1α expression was also induced by exposure for 1 h to IL-1β and Rb_1 in HaCaT cells (Figure 7b). Ginsenoside Rb_1 did not enhance the HIF-1α expression in HaCaT cells exposed to IL-1β for 2 h (data not shown).

*Effect of ginsenoside Rb_1 on cell proliferation and cytotoxicity in HaCaT cells (*in vitro*)*

Ginsenoside Rb_1 did not cause cytotoxicity in HaCaT cells at 100 fg ml^{-1}, 10 pg ml^{-1} or 1 ng ml^{-1} (Table 4). Furthermore, ginsenoside Rb_1 had no effect on the cell proliferation of HaCaT cells.

Discussion

It has been reported that burn injury induces inflammatory and immune dysfunction (Alexander & Moncrief, 1966; Faunce *et al*., 1997; Leder *et al*., 1999; Ramzy *et al*., 1999). In particular, the acute response to burn injury results in a coordinated influx of leukocytes such as PMNs and macrophages at the wound site. It is well known that VEGF induces the migration and proliferation of endothelial cells and enhances vascular permeability, consistent with its purported ability to promote angiogenesis (Connolly *et al*., 1989; Gospodarowicz *et al*., 1989; Keck *et al*., 1989; Leung *et al*., 1989). Therefore, VEGF plays a crucial role in tissue repair, as angiogenesis and increased vascular permeability are important during wound healing (Brown *et al*., 1992; Nissen *et al*., 1998). Frank *et al*. (1995) reported that VEGF might promote wound healing, as shown by a study of genetically diabetic mice (*db*/*db* mice), in which VEGF expression fails at the

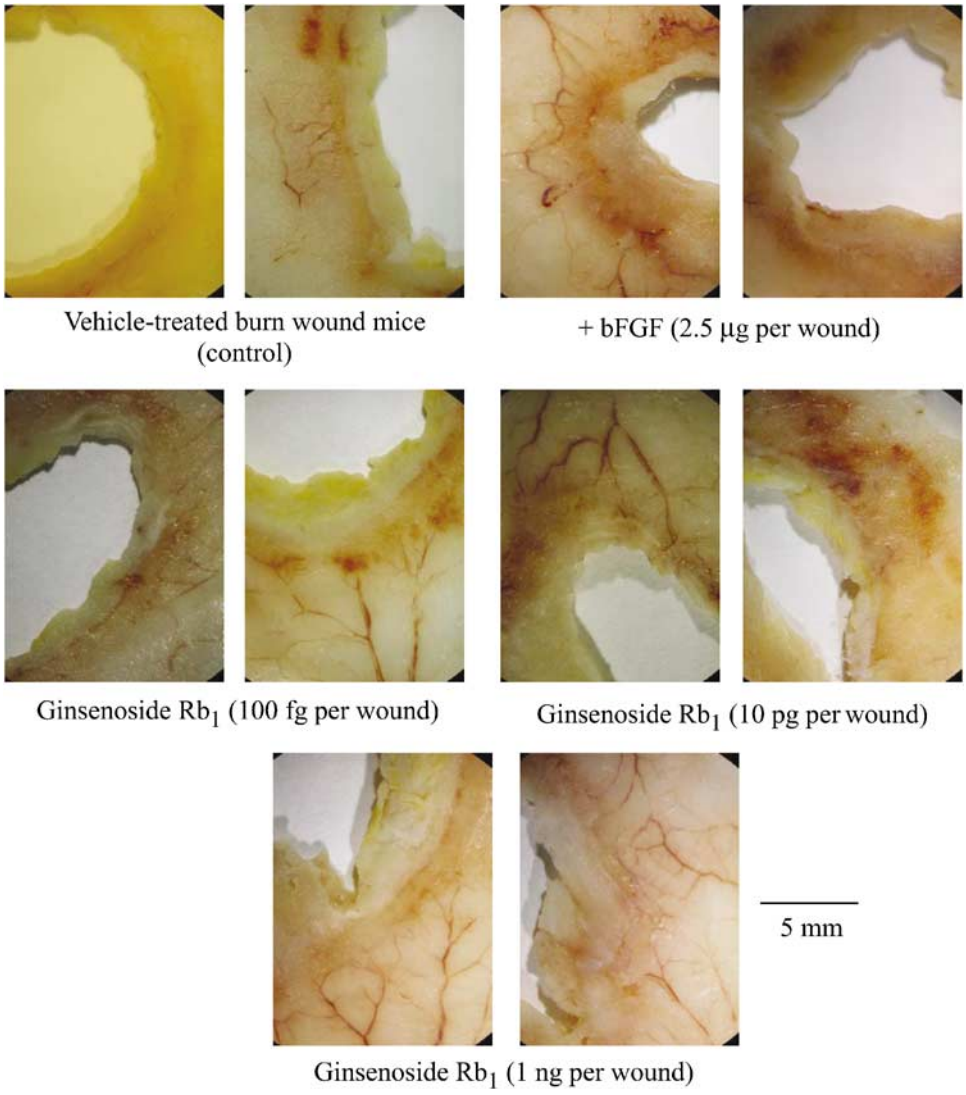


Figure 5 Photographs showing neovascularization from the tissue surrounding the burn wound area and the effects of the topical application of ginsenoside Rb_1 (100 fg, 10 pg and 1 ng per pellet) and bFGF (2.5 μg per pellet).

Table 2 Effects of ginsenoside Rb_1 on neovascularization from surrounding burn wound area in mice

	No. of animals	*Blood vessel length (mm per field)*	*Blood vessel area (mm^2 per field)*
Untreated burn wounds (control)	6	75.57±24.93	10.49±3.80
+Ginsenoside Rb_1			
(100 fg per pellet)	6	228.80±38.60*	46.56±15.01*
(10 pg per pellet)	6	203.00±17.01*	37.673±5.51*
(1 ng per pellet)	6	274.08±37.39*	49.94±4.70*
+Basic FGF			
(2.5 μg per pellet)	6	241.46±28.30*	35.80±5.86*

Values are means±s.e. of six mice. *Significantly different from untreated burn wounds (control), $P<0.05$.

wound site and healing is impaired (Coleman, 1982; Frank *et al*., 1995). Therefore, they suggested that keratinocyte-derived VEGF might stimulate angiogenesis during wound healing.

The genus *Panax* derives its name from the Greek *pan* (all) and *akos* (healing), as named by the botanist Carl Meyer. Kanzaki *et al*. (1998) reported that orally administered Red Ginseng root stimulated the repair of intractable skin ulcers in patients with diabetes mellitus and Werner's syndrome in clinical trials, and that local administration of saponin markedly improved wound healing in diabetic or aging rats (Morisaki *et al*., 1995). Recently, Sengupta *et al*. (2004) reported that ginsenoside Rg_1 promoted functional neovascularization into a polymer scaffold *in vivo* and the proliferation and chemoinvasion of tube-like capillary formation by human umbilical vein endothelial cells (HUVECs) *in vitro* through

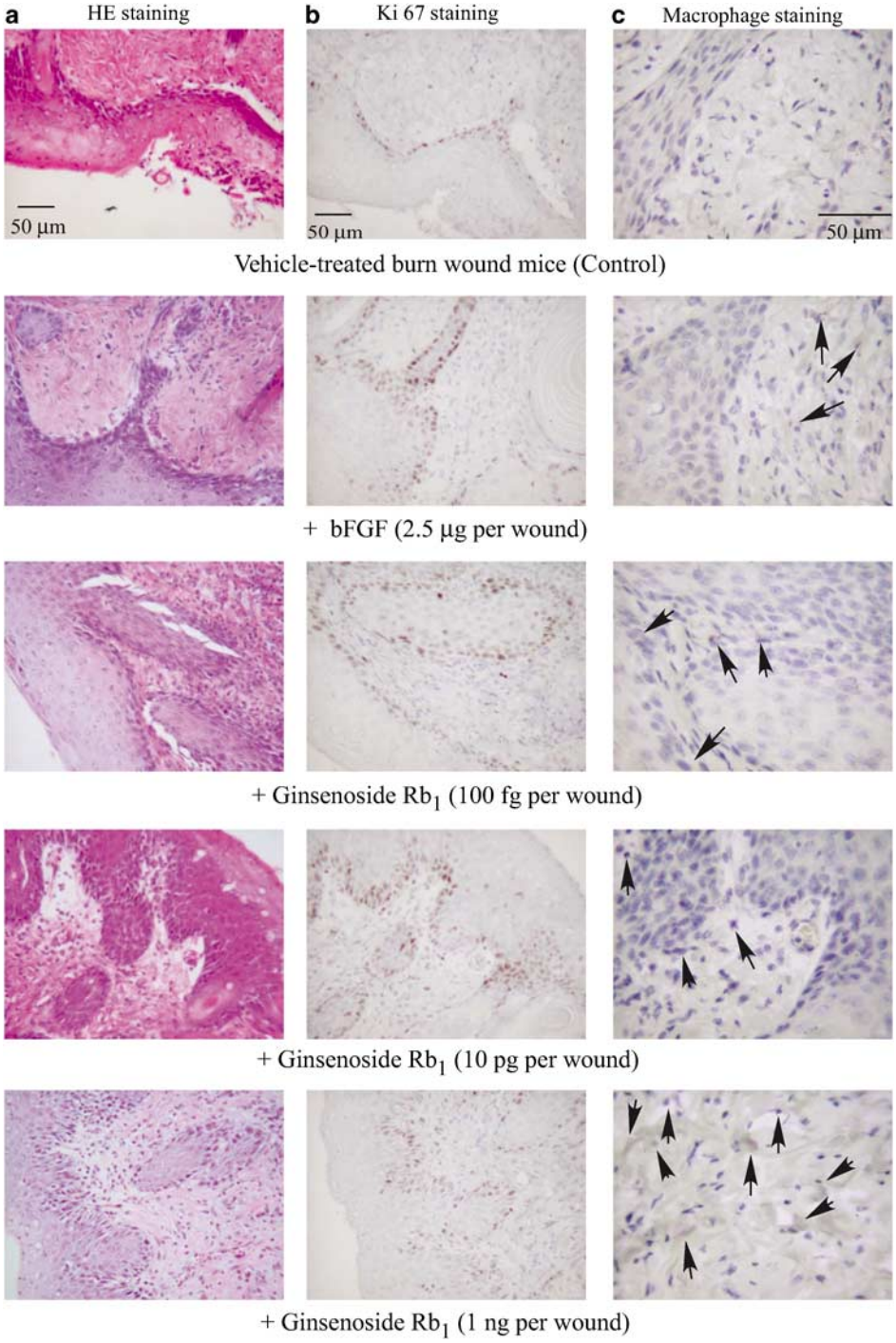


Figure 6 Light micrographs of cells stained with hematoxylin–eosin (HE) (× 200 magnification) (a), anti-mouse Ki-67 rat monoclonal antibody to show the keratinocyte proliferation (× 200 magnification) (b) or stained with anti-macrophage mouse monoclonal antibody to show the macrophage migration (× 400 magnification) (c). Antibody-treated samples were stained by the immunoperoxidase technique and counterstained with hematoxylin. Arrow denotes macrophages.

enhanced expression of nitric oxide synthetase, phosphatidylinositol-3 kinase and the Akt pathway. On the other hand, ginsenoside Rb_1 inhibited the earliest step in angiogenesis, the chemoinvasion of HUVECs (Sengupta *et al*., 2004). Furthermore, among the various ginsenosides, ginsenoside Rb_2 has been reported to improve wound healing through its enhancing effects on epidermal cell proliferation, by upregulating the expression of proliferation-related factors (Choi, 2002). On the other hand, it has been reported that intravenous administration of ginsenoside Rb_2 inhibited metastasis to the lung through the inhibition of tumor-induced neovascularization in B16-BL6 melanoma-bearing mice (Sato *et al*., 1994).

To clarify these perplexing contradictions in the reported effects of various ginsenosides on wound healing, we have examined the effects of total ginseng saponins and various ginsenosides on healing of burn wounds in mice. We found

that ginsenoside Rb_1 was the most potent agent for wound healing among the various ginsenosides we used. Furthermore, we examined the effects of ginsenoside Rb_1 on Ki-67 expression, on the accumulation of macrophages and on the production of cytokines (VEGF and IL-1β) in the regenerating skin area of burn wounds. We found that ginsenoside Rb_1, at doses of 100 fg–1 ng per wound area, significantly increased the neovascularization from the tissue surrounding the burn wound area as compared to controls; VEGF and IL-1β production from the wound area and the macrophage accumulation were all significantly increased as well, compared

Table 3 Effects of ginsenoside Rb_1 on the number of Ki-67-positive cells in regenerating skin of burn wounds in mice

	No. of animals	*Ki-67-positive cells (number per field)*
Untreated burn wounds (Control)	6	94.0±25.9
+Ginsenoside Rb_1		
(100 fg per pellet)	6	249.7±28.0*
(10 pg per pellet)	6	198.8±26.7*
(1 ng per pellet)	6	286.5±31.3*,#
+Basic FGF (2.5 μg per pellet)	6	204.7±13.5*

Values are means±s.e. of six mice. *Significantly different from untreated burn wounds (control), $P<0.05$; #Significantly different from bFGF-treated wounds, $P<0.05$.

Table 4 Effects of ginsenoside Rb_1 on cell proliferation and cytotoxicity in HaCaT cells in culture

	% of control
Medium alone (control)	100
+Ginsenoside Rb_1	
(100 fg ml^{-1})	107±7.9
(10 pg ml^{-1})	101±1.4
(1 ng ml^{-1})	100±4.7

Values are means±s.e. of four experiments.

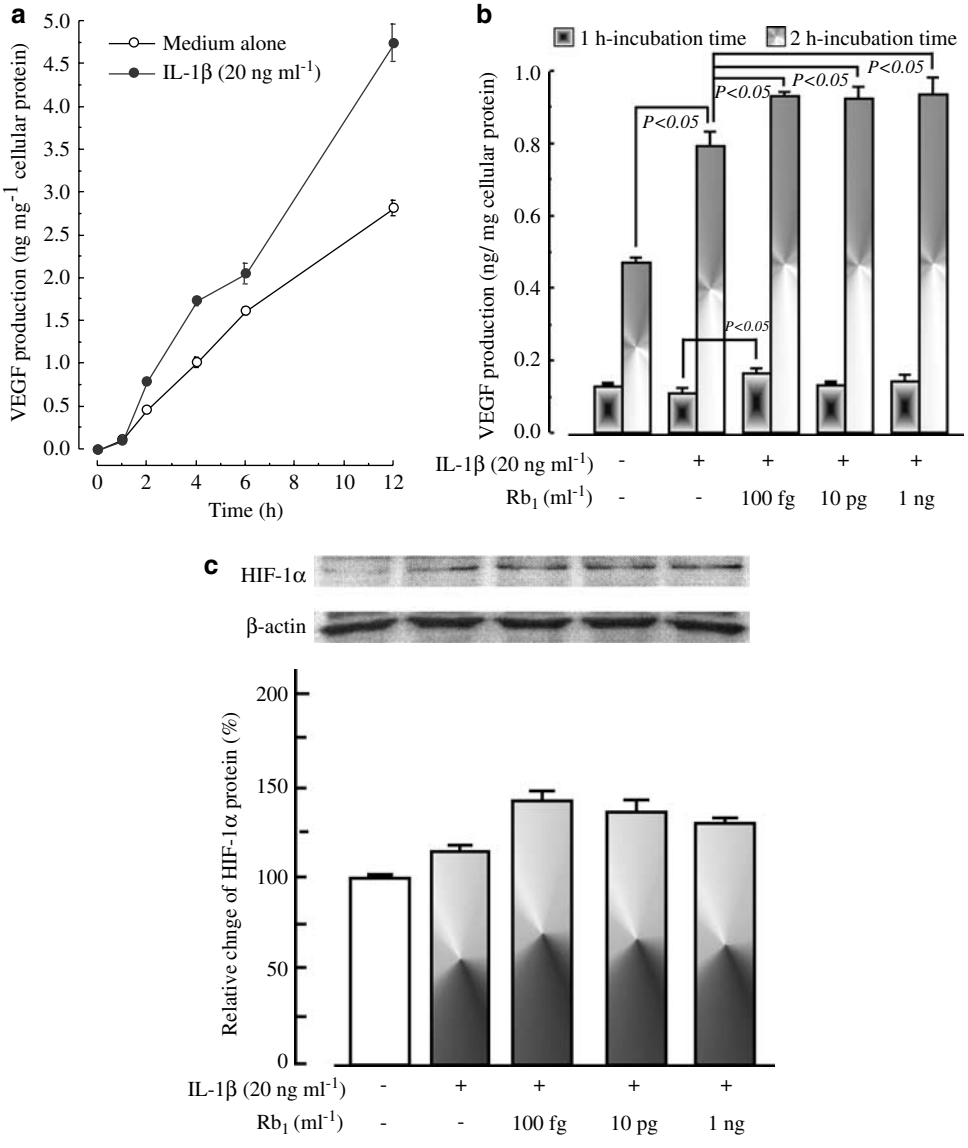


Figure 7 Effects of ginsenoside Rb_1 on VEGF production (a, b) and HIF-1α protein expression (c) with or without IL-1β in cultured HaCaT cells. Values are means±s.e. of six experiments.

to those in control mice. Further studies are now in progress to clarify the enhancing effects of ginsenoside Rb_1 on macrophage migration to the wound site and the wound-healing process.

In addition, we attempted to clarify some mechanistic aspects of the effects of ginsenoside Rb_1 on VEGF production in a human keratinocyte cell line, HaCaT cells. Ginsenoside Rb_1 at concentrations from 100 fg ml^{-1} to 1 ng ml^{-1} enhanced the VEGF production and HIF-1α expression induced by IL-1β in HaCaT cells. HIF-1 is a heterodimeric complex composed of α and β subunits. The β subunit is constitutively expressed and is identical to aryl hydrocarbon nuclear translocator. The HIF-1α subunit is specifically induced by hypoxia and decays rapidly in normoxia (Wang *et al*., 1995). HIF-1α binds to specific promoter moieties of genes encoding erythropoietin, VEGF, glycolytic transporters (Ben-Av *et al*., 1995; Wenger & Gassmann, 1997; Paleolog *et al*., 1998) and inflammatory mediators (Faquin *et al*., 1992; Hellwig-Bügel *et al*., 1999; Thornton *et al*., 2000). It has been reported that IL-1β stimulates VEGF and HIF-1α expression during inflammation (Ben-AV *et al*., 1995; Hellwig-Bügel *et al*., 1999; Thornton *et al*., 2000), and that IL-1, in both the IL-1α and the IL-1β forms, induces transcription of keratin K6 in human epidermal keratinocytes (Komine *et al*., 2001). Therefore, the findings in the present study suggest that the enhancement of burn wound healing by ginsenoside Rb_1 might be owing to the promotion of angiogenesis during skin wound repair as a result of the stimulation of VEGF production caused by the increase of HIF-1α expression in keratinocytes. The increase in HIF-1α expression results from the stimulation of IL-1β production owing to the increased macrophage accumulation in the burn wound site. Basic FGF, used as a positive control, also promotes burn wound healing at a concentration of 2.5 μg per mouse through the enhancement of angiogenesis *via* the increase in VEGF and IL-1β production.

We found that ginsenoside Rb_1 promotes burn wound healing and was the most active of the six ginsenosides tested here, being effective at very low concentrations (100 fg to 1 ng per mouse). Sengupta *et al*. (2004) have reported that ginsenoside Rb_1 (125 nM, 140 ng ml^{-1}) reduced hepatocyte growth factor-induced HUVEC migration, and accelerated the proliferation of HUVEC at 0.125, 1.25 and 125 nM (0.14, 1.4 and 140 ng ml^{-1}). The concentrations of Rb_1 used by Sengupta *et al*. (2004) are greater than those used in the present study (100 fg, 10 pg and 1 ng per wound or per ml), *in vivo* or *in vitro*. Recently, we reported that intravenous infusion of ginsenoside Rb_1 at low doses (6 or 60 μg day^{-1}), prevented ischemic neuronal death through the upregulation of Bcl-x_L expression, but at high doses (3 or 12 mg day^{-1}) it had no effect (Zhang *et al*., 2005). Thus, the pharmacological actions of ginsenoside Rb_1 are exhibited at low doses, and there are no significant differences among the low doses of ginsenoside Rb_1. The reason for this is unknown; therefore, further studies are needed. Further experiments will also be needed to clarify the clinical significance of these findings for wound healing.

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