Modulating Angiogenesis
The Yin and the Yang in Ginseng

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Background—Ginseng is a commonly used nutraceutical. Intriguingly, existing literature reports both wound-healing and antitumor effects of ginseng extract through opposing activities on the vascular system. To elucidate this perplexity, we merged a chemical fingerprinting approach with a deconstructional study of the effects of pure molecules from ginseng extract on angiogenesis.

Methods and Results—A mass spectrometric compositional analysis of American, Chinese and Korean, and Sanqi ginseng revealed distinct “sterol ginsenoside” fingerprints, especially in the ratio between a triol, Rg1, and a diol, Rb1, the 2 most prevalent constituents. Using a Matrigel implant model and reconstituting the extracts using distinct ratios of the 2 ginsenosides, we demonstrate that the dominance of Rg1 leads to angiogenesis, whereas Rb1 exerts an opposing effect. Rg1 also promoted functional neovascularization into a polymer scaffold in vivo and the proliferation of, chemoinvasion of, and tubulogenesis by endothelial cells in vitro, an effect mediated through the expression of nitric oxide synthase and the phosphatidylinositol-3 kinase→Akt pathway. In contrast, Rb1 inhibited the earliest step in angiogenesis, the chemoinvasion of endothelial cells.

Conclusions—The present study explains, for the first time, the ambiguity about the effects of ginseng in vascular pathophysiology based on the existence of opposing active principles in the extract. We also unraveled a speciﬁcographic variation impinging on the compositional fingerprint that may modulate the ﬁnal phenotype. This emphasizes the need for regulations standardizing herbal therapy, currently under the Dietary Supplement and Health Education Act. Furthermore, we propose that Rg1 could be a prototype for a novel group of nonpeptide molecules that can induce therapeutic angiogenesis, such as in wound healing. (Circulation. 2004;110:1219-1225.)

Key Words: ginsenosides □ angiogenesis □ nitric oxide

The use of botanicals as alternative medicine has seen a resurgence of use in the United States and Europe. In 2001, $17.8 billion was spent on dietary supplements, $4.2 billion of it for herbal remedies.1 It is estimated that >12.1% of adults in the United States used herbal medicines in 1997.2 Interestingly, in the United States, herbal medicines are regulated as dietary supplements and fall under the purview of the 1994 Dietary Supplement and Health Education Act (DSHEA) and thus can be marketed without prior approval by the Food and Drug Administration (FDA).3

Ginseng is a key component in traditional Chinese medicine and is also one of the most extensively used botanical products in the West, which is evident from annual sales of US $0.225 to $0.3 billion.4 Named by the botanist Carl Meyer, the genus Panax derives its name from the Greek pan (all) and akos (healing).5 The activity of ginseng extract has been studied extensively. Ambiguously, ginseng extract has been reported to promote wound healing6 and inhibit tumor growth7 through opposing effects on angiogenesis. To elucidate this perplexity in the reported effects of ginseng, we merged a chemical fingerprinting approach with a deconstructional study of the angiogenic process and identified for the first time the existence of active principles in the extract that can exert opposing effects on the final phenotype. On the basis of our present findings, we call for stronger regulations controlling the use of herbal drugs.

Received April 9, 2003; de novo received January 2, 2004; revision received February 19, 2004; accepted April 16, 2004.

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The online-only Data Supplement, which contains additional information about Methods used in this study, is available at http://www.circulationaha.org.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000140676.88412.CF
Methods

Mass Spectrometric Chemical Fingerprinting
Four different varieties of ginseng—Chinese and Korean ginseng (Panax ginseng), American ginseng (P quinquefolium), and Sanqi ginseng (P notoginseng, Yunnan, China)—were collected and subjected to methanolic extraction. The fractions were qualitatively analyzed with the use of an ESI mass spectrometer (Perkin-Elmer Sciex API 365 Tandem spectrometer), and the mass spectrometry spectra were acquired in a positive scan mode.

Chemo invasion Assay
Synchronized human umbilical vein endothelial cells (HUVECs) were plated on the upper chamber of Matrigel-coated transwells (8-μm pore diameter, Costar), at a density of 10^4 cells/well. The drugs and growth factors were added in the appropriate chambers, and the cells were incubated for 16 hours, at which time the cells on the upper side of the membrane were wiped off. The cells that had migrated to the lower side of the membrane were fixed in 10% formalin, stained with hematoxylin-eosin, and visualized with the ×20 objective lens of a Nikon Diaphot inverted microscope.

Endothelial Healing
A 24-well plate containing a semicircular coverslip in each well, overlaid with 0.1% gelatin, was seeded with HUVECs at a density of 3×10^4 cells/well in supplemented M199 media with 20% (vol/vol) FCS. On reaching confluence, the cells were synchronized for 24 hours, after which the half coverslips were removed, leaving a sharp margin of cells adjacent to the wound edge. The cells were incubated for 48 hours in the presence of appropriate test agents in M199 media with 1% (vol/vol) FCS, after which they were harvested and counted with a hemocytometer.

Western Blot Analysis
Cells were treated as described above and lysed in sample buffer (×3) under reducing conditions. Equivalent amounts of protein per sample were electrophoretically resolved on 10% SDS-PAGE and transferred to membrane. Proteins were detected with the use of antibodies directed to endothelial nitric oxide synthase (NOS) (1:250 dilution; New England BioLabs). Anti–β-actin antibodies (Sigma) were used at a 1:1,000 dilution to normalize the loading of proteins. Activation of Akt was detected with an antibody specific for phospho-Akt (1:800 dilution; Cell Signaling Technology), and the membrane was reprobed with an antibody against total Akt to normalize. Membranes were incubated with a 1:2,000 dilution of the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham). The immunocomplexes were visualized with the use of enhanced chemiluminescence detection (Amersham).

In Vivo Matrigel Angiogenesis Assay
Growth factor–reduced Matrigel (Becton Dickinson), mixed with growth factor and/or drugs, was injected subcutaneously into male C57/BL6 mice. On day 9, the animals were euthanized by CO2 exposure, the skin was everted, and Matrigel were excised. The implants were excised and cryofrozen in OTC for immunohistochemistry. The Matrigel sections (12 μm) were fixed in −20°C methanol and probed with antibody against von Willebrand factor (vWF). The signal was amplified with a Texas red–labeled secondary antibody, and the images were captured with a Zeiss LSM510 confocal microscope. Propidium iodide was used to counterstain nuclei.

In Vivo Scaffold Implant Neovascularization Assay
Male Balb/c mice (20 to 22 g body wt; Tuck, Rayleigh, UK) were anesthetized with isoflurane in a mixture of oxygen (0.8 L/min) and nitrous oxide (0.6 L/min). Two bilateral subcutaneous air pockets were fashioned on the dorsum. A sterile polyether polyurethane sponge (170 mm3) was inserted into each pocket, and the incision was closed. The treatment with vehicle or drug was started 24 hours after implantation by direct injection into the sponge once daily for 10 days. On day 15 after implantation, the animals were euthanized by cervical dislocation. Neovascularization was quantified as the ingrowth of vessels in the granuloma tissue and photographed with a macro-lens (Nikon). All studies were approved and performed under guidelines set by the home office in the United Kingdom.

Quantification of the Functionality of Neovessels
Functionality of the vessels was assessed in terms of the clearance of radioactive 133Xe from the sponge implant. On day 15 after sponge implantation, the animals were anesthetized with a combination of Hypnorm (Janssen Pharmaceutica) and Hypnovel (Roche). The clearance of 133Xe-containing saline was monitored over a 6-minute period. Radioactivity was measured with a microprocessor scalar rate meter (Nuclear Enterprise), linked to a low-energy x-ray/γ-ray NaI crystal with an aluminum entrance window, on an HG-type mount coupled to an NE 5280c preamplifier. Data were expressed as the percentage of 133Xe cleared at 6 minutes relative to that at the time of administration.

Immunohistological Staining for Vasculature
Cryostat sections (10 μm) were incubated overnight with a polyclonal rabbit antibody against human vWF (1:2000 dilution; Dako) in blocking solution (1% [vol/vol] FCS in Tris-buffered saline with Tween). Antibody binding sites were visualized with the use of a goat anti-rabbit antibody conjugated to FITC (1:150 dilution; Vector Labs). The fluorochrome was excited with the use of a 488-nm laser line, and the emitted light was captured with the use of a 530/30-nm bandpass filter. The images were captured at a resolution of 512×512 pixels with a Leica TCS-NT confocal microscope. Controls were run alongside by omitting the primary antibody.

Statistical Analysis
Data are expressed as mean±SEM. Statistical significance was tested with 1-way ANOVA followed by the Dunnett or Friedman post hoc test. The Bonferroni test was used to test for overall dose-response effects (Graphpad Prism 3 software). P<0.05 was considered significant.

Results

Compositional Analysis
We obtained 4 different varieties of ginseng, covering the geographic distribution and species variation—Chinese and Korean ginseng (P ginseng), American ginseng (P quinquefolium), and Sanqi ginseng (P notoginseng, Yunnan, China)—and subjected the methanolic extract from each to a mass spectrometric analysis (Figure 1A to 1D). The active components of ginseng are triterpene dammarenes, better known as ginsenosides, and they can be classified as panaxdiols or panaxtriols on the basis of their structures. The most common of the diols and triols were Rb1 and Rg1, respectively, which exhibit structural similarities in the unique diols or panaxtriols on the basis of their structures. The most common of the diols and triols were Rb1 and Rg1, respectively, which exhibit structural similarities in the unique steroid backbone but are different at the level of side chains and hydroxyl substitution (Figure 1E and 1F). The percent composition (wt/wt) of Rg1 and Rb1 in each species was quantified with a reverse-phase HPLC (Figure 2) and is shown in the Table in the online-only Data Supplement. Both Rg1 and Rb1 were present in all the extracts; however, interestingly, each extract displayed a unique ginsenoside mass spectrometric fingerprint, especially in the ratio between the two (online-only Data Supplement Table).

Effect on Chemo invasion
An activated endothelial cell usually mounts an angiogenic phenotype through coordinated sequential steps. One of the
earliest and key steps during neovascularization is the invasion of the basement membrane and migration of the endothelial cells toward an appropriate cue, usually a growth factor or energy source. We mimicked this effect in vitro using a chemoinvasion assay, in which the HUVECs were stimulated to invade through an extracellular matrix and migrate toward an angiogenic chemoattractant. We used hepatocyte growth factor/scatter factor (HGF/SF), a tyrosine kinase receptor ligand, as a chemoattractant in this study, and we have previously demonstrated that it is a potent and independent activator of the endothelium.\(^9\)\(^{10}\) In this assay, although neither Rg1 nor Rb1 exerted any independent chemoattractant property, Rb1 inhibited the HGF/SF-induced chemoinvasion. In contrast, Rg1 was found to potentiate the chemoinvasion by the endothelial cells toward HGF/SF. Furthermore, the Rg1-treated cells were observed as aligned in a cordlike fashion, mimicking a physiological vasculature, on the lower side of the chamber (Figure 2).

**Endothelial Healing In Vitro**

Endothelial cells are normally contact inhibited physiologically and attain an aggressive phenotype only when this inhibition is destabilized. To best mimic this switch in vitro, we measured the effects of Rg1 and Rb1 on the proliferation of HUVECs in a monolayer injury assay. As shown in Figure 3, both the molecules exerted a concentration-dependent
proliferation of HUVECs compared with the vehicle-treated controls. This was a novel observation in that ginsenosides are sterol compounds, and it was expected that they would inhibit proliferation like the other angiostatic steroids and glucocorticoids, which are known to act through the nuclear receptors to impinge at a transcriptional level.

In Vivo Effects of Ginsenosides

On the basis of the outcome of the in vitro experiments, we evaluated the effects of the ginsenosides on angiogenesis in an avascular polyether polyurethane scaffold implant in mice. This model has been standardized extensively for studying morphological and functional neovascularization. The molecules were injected at a dose of 10 μg, intrascaffold, for 10 days, and the angiogenic outcome was quantified as the radial ingrowth of vessels into the scaffold granuloma tissue. Functionality of the neovascularure was quantified by measuring the clearance of radioactive 133Xe from the implant, in which a faster clearance denotes active blood flow.

As shown in Figure 4, Rg1 produced a strong functional neovascularization in the scaffold implants that was corroborated by immunostaining for vWF, a marker for endothelial cells. This observation was in agreement with the findings of in vitro studies in which Rg1 was found to stimulate all the sequential steps to an overt phenotypic neovascular outcome. In contrast, Rb1, at the dose administered, failed to exert any detectable effect in vivo, consistent with the in vitro effects of Rb1 (shown in Figure 4) in that it had inhibited the chemoinvasion of endothelial cells or essentially the initial step in neovascularization.

To evaluate the significance of the distinct compositional ratio of Rg1 and Rb1 in the extracts of ginseng species, we reconstituted defined extracts with a defined ratio between Rg1 and Rb1 and evaluated the effect on angiogenesis in a Matrigel implant murine model. As shown in Figure 5, the administration of an extract with a greater percentage of Rg1 than Rb1 resulted in the induction of significant angiogenesis into the implant compared with vehicle-treated implants. In

Figure 2. Effect of ginsenosides on chemoinvasion by HUVECs. Photomicrographs depict cells migrated under control conditions (A) and HGF/SF (1 nmol/L)–facilitated HUVEC chemoinvasion and spreading. C, Rg1 treatment increases the HGF/SF-induced chemoinvasion and spreading of HUVECs. D, Rb1 (125 nmol/L) inhibited the HGF/SF-induced chemoinvasion. Right, Mean number of migrated cells per high-power field under different experimental conditions (mean±SEM; n=4). *P<0.05, **P<0.001 vs HGF/SF-induced chemoinvasion. Upper and lower indicate the 2 chambers of the transwell; Veh, vehicle. Arrow depicts a typical cell that has migrated to the lower side and spread.

Figure 3. Effect of Rg1 and Rb1 on the proliferation of HUVECs, after a mechanical injury to the monolayer, and modulation by NOS inhibitors. The cells were harvested and counted after 48-hour incubation in the presence of Rg1 (A) and Rb1 (B). C, The NOS inhibitor L-NAME inhibited the Rg1-induced proliferation of HUVECs in a concentration-dependent manner. At 10^{-3} mol/L, L-NAME did not alter the basal response. D, L-NAME inhibited the Rb1-induced proliferation at higher concentrations. The cells were incubated in the presence of L-NAME (LN) (10^{-2} mol/L), increasing concentrations of Rg1 (E), and increasing concentrations of the NOS substrate L-arginine (L-Arg) (F). G, Effects of NOS-III inhibitor L-NIO and NOS-II inhibitor SMT on Rg1-induced cell proliferation. H, Increasing concentrations of Rg1 can overcome the inhibitory effects of L-NIO. Values at 0 hour denote the cell count immediately after wounding. +P<0.01 vs Rb1 or Rg1 alone; *P<0.05, **P<0.01, ***P<0.001 vs vehicle (veh)-treated controls. Data are mean±SEM from 3 to 6 experiments with duplicate wells in each.
contrast, the overabundance of Rb1 inhibited the Rg1-induced neovascularization.

**Discussion**

The present study addresses for the first time the controversial role of ginseng in wound healing and tumor growth by elucidating the mechanistically opposing activity exerted by the 2 key ginsenosides on the angiogenic phenotype. Furthermore, we also demonstrate a speciogeographic variation impinging on the compositional fingerprint that may alter the phenotypic outcome (for additional HPLC compositional analysis, see the online-only Data Supplement [particularly Data Supplement Figure 1]). Indeed, the extract from *Panax* ginseng, which has a predominance of Rb1, exerted a preventive effect in multiple cancer models. Similarly, American ginseng was found to synergize with breast cancer chemotherapeutic agents, coinciding with the interpretation of our data. In contrast, Sanqi ginseng is considered the key ingredient for the treatment of trauma injuries and for promoting microcirculation, which would be consistent with the proangiogenic activity of its predominant ginsenoside Rg1.

The activity of Rg1 at both in vivo and in vitro levels opened up the exciting possibility of dissecting out the downstream signal cascades of Rg1. We used pharmacological inhibitors to study the effects of Rg1 and Rb1 on mitogen-activated protein kinases and NO signaling, 2 key pathways that have been implicated in phenotypic outcome described for the endothelial cells. Neither Rg1 nor Rb1 had any effect on activation of the mitogen-activated protein kinase pathway (data not shown). Intriguingly, the treatment with Rg1 induced a strong expression of NOS-III protein in the cells after 24 hours (Figure 6). Furthermore, the Rg1-induced proliferation of HUVECs was abolished by N^G^-nitro-L-arginine methyl ester (L-NAME), a nonselective inhibitor of NOS (Figure 3C), indicating that the proliferative effects of Rg1 are NO dependent (Figure 3C). The inhibitory effect of L-NAME on the Rg1-induced proliferation was partially overcome by the NOS substrate L-arginine, which competes with L-NAME for the active site (Figure 3E). Higher concentrations of Rg1 completely reversed the block (Figure 3F). It has been demonstrated recently that phosphatidylinositol 3 kinase (PI3K) and Akt lie upstream and can activate NOS-III. Rg1 also induced the phosphorylation of Akt, which was blocked by the PI3K inhibitor LY294002 (Figure 6). These observations suggest that in addition to promoting the synthesis of NOS enzyme, Rg1 can activate through the PI3K→phospho-Akt→NOS pathway.

NO has been implicated in the induction of neovascularization. However, Pipili-Synetos et al reported that NO donors inhibit the aggressive endothelial phenotype and neovascularization in the chick chorioallantoic membrane assay. They subsequently demonstrated that this inhibition in the chorioallantoic membrane assay is paralleled by an increase in NOS-II expression and activity. However, in the present study, the selective NOS-II inhibitor SMT did not affect the Rg1-induced proliferation, nor could we detect it using Western blots. On the contrary, the NOS-III inhibitor L-NIO blocked the Rg1-induced cell proliferation (Figure 3G), and a reversal was effected by increasing concentrations of Rg1 (Figure 3H), implying that the NOS-III isoform may be playing the key role in the Rg1-induced activation of the...
endothelium. The coadministration of L-NAME with Rg1 blocked the angiogenic effect of the latter (Figure 4) in vivo. This result attains significance because NOS-III has been implicated in neovascularization related to wound healing.23 Furthermore, the NO-mediated effects of Rg1 could possibly account for the potentiation of the tubulogenic effects of vascular endothelial growth factor and fibroblast growth factor-2 on the endothelium (see online-only Data Supplement [Data Supplement Figure 2]) because these ligands induce neovascularization through a NO-dependent signal cascade.20

Intriguingly, although coinubcation with L-NAME completely blocked the Rg1-induced tubulogenesis, it failed to exert a similar effect on Rb1. Similarly, Rb1-induced endothelial cell proliferation was only partially inhibited by L-NAME, suggesting that additional pathways could be mediating the effects of Rb1. In a recent study, Rb1 was shown to inhibit tyrosine kinase,24 which could explain its inhibition of HGF/SF-induced chemoinvasion. Such differences at an intracellular signaling level could possibly exert a bearing in the divergence in the phenotypic outcome at the level of the chemoinvasive property of endothelial cells and govern the final global outcome in a pathophysiological setting.

The marked spatiotemporal distinction in the mode of action of ginsenosides opens up the exciting possibility of developing newer nonpeptide small molecule–based therapeutic approaches for the management of impaired endothelial function in a pathophysiological context. Whereas further studies could reveal the chemopreventive role for Rb1-like molecules in inhibiting pathological neovascularization, such as in cancer and diabetic retinopathy, the identification of Rg1 as a proactive modulator opens up the exciting possibilities of harnessing the chemical scaffold as a prototype for wound-healing compounds. Although this is exciting, our present study also raises a note of caution because we observed that reconstituting an extract by adding Rg1 and Rb1 in a defined ratio could alter the angiogenic outcome. Furthermore, our own analysis and those of others25 revealed that not only can the species and regional variation impinge on the compositional ratio, but the way the extracts are processed for commercial use can alter the fingerprint as well (data not shown). Intriguingly, according to the DSHEA, the manufacturers of dietary supplements are responsible for the truthfulness of any claims and must provide evidence for the claims, yet it neither provides a standard for the evidence needed nor requires the submission of the evidence to the FDA.1 The perception that herbal remedies, used as folk medicine, are inherently safe and efficacious is based on traditional “knowledge” rather than systematic studies, and recent findings have spurred calls for quality, safety, and efficacy assurance.3 The present findings about the existence of opposing active principles in one of the most prevalent herbal medicines emphasizes the importance of standardiza-
tion through compositional analysis and calls for better regulation.

Acknowledgments
This study was supported by Hong Kong RGC (Drs Fan and Wong), Cambridge Nehru Trust and CVCP (UK) (Dr Sengupta), the Wellcome Trust (Dr Skepper), and the Dutch Cancer Society (grant TNOP-97-1511) (Dr Koolwijk). The authors thank Erna Peters for her excellent technical assistance.

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Circulation. 2004;110:1219-1225; originally published online August 30, 2004;
doi: 10.1161/01.CIR.0000140676.88412.CF
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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Table 1.

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<tr>
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Concentrations w/w%:

- *Panax notoginseng*: 2.176  2.331
- *Panax quinquefolium L.*: 0.051  2.510
- *Panax ginseng*: 0.213  0.515
Supplementary Figure 2