

DERMAL WOUND HEALING PROPERTIES OF REDOX-ACTIVE GRAPE SEED PROANTHOCYANIDINS

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(Received 7 March 2002; Revised 11 June 2002; Accepted 20 June 2002)

Abstract—Angiogenesis plays a central role in wound healing. Among many known growth factors, vascular endothelial growth factor (VEGF) is believed to be the most prevalent, efficacious, and long-term signal that is known to stimulate angiogenesis in wounds. The wound site is rich in oxidants, such as hydrogen peroxide, mostly contributed by neutrophils and macrophages. We proposed that oxidants in the wound microenvironment support the repair process. Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Previously we have reported that a grape seed proanthocyanidin extract containing 5000 ppm resveratrol (GSPE) potently upregulates oxidant and tumor necrosis factor- α inducible VEGF expression in human keratinocytes (*Free Radic. Biol. Med.* 31:38–42, 2001). Our current objective was to follow up on that finding and test whether GSPE influences dermal wound healing in vivo. First, using a VEGF promoter-driven luciferase reporter construct we observed that the potentiating effect of GSPE on inducible VEGF expression is at the transcriptional level. The reporter assay showed that GSPE alone is able to drive VEGF transcription. Next, two dermal excisional wounds were inflicted on the back of mice and the wounds were left to heal by secondary intention. Topical application of GSPE accelerated wound contraction and closure. GSPE treatment was associated with a more well-defined hyperproliferative epithelial region, higher cell density, enhanced deposition of connective tissue, and improved histological architecture. GSPE treatment also increased VEGF and tenascin expression in the wound edge tissue. Tissue glutathione oxidation and 4-hydroxynonenal immunostaining results supported that GSPE application enhanced the oxidizing environment at the wound site. Oxidants are known to promote both VEGF as well as tenascin expression. In summary, our current study provides firm evidence to support that topical application of GSPE represents a feasible and productive approach to support dermal wound healing. © 2002 Elsevier Science Inc.

Keywords—Wound healing, Angiogenesis, Skin, Redox, Tannins, Oxidant

INTRODUCTION

Angiogenesis plays a central role in wound healing. Among many known growth factors, vascular endothelial growth factor (VEGF) is believed to be the most prevalent, efficacious, and long-term signal known to stimulate angiogenesis in wounds [1]. VEGF is a homodimeric glycoprotein that is highly conserved and shares structural homology with placenta growth factor

and platelet-derived growth factor [2,3]. It induces migration and proliferation of endothelial cells and enhances vascular permeability [4] consistent with the purported ability to promote angiogenesis.

Inflammation, constituting part of the acute response, results in a coordinated influx of neutrophils at the wound site. These cells, through their characteristic “respiratory burst” activity, produce $O_2^{\bullet-}$, which is very well known to be critical for defense against bacteria and other pathogens [5]. The production of oxidants at the wound site is not restricted to neutrophils alone but may also be produced by macrophages, which appear and orchestrate a “long-term” response to injured cells subsequent to the acute response. Wound-related nonphago-

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cytic cells also generate $O_2^{\bullet-}$ by a nonphagocytic NAD(P)H oxidase or Nox-dependent mechanism [6]. Superoxide anion radical is rapidly converted to membrane permeable form, H_2O_2 , by superoxide dismutase activity or even spontaneously. Release of H_2O_2 may promote formation of other oxidant species, including hypochlorous acid, chloramines, aldehydes, etc. Taken together, this suggests that the wound site is rich in both oxygen- and nitrogen-centered reactive species along with their derivatives (e.g., H_2O_2 , $O_2^{\bullet-}$, $\cdot NO$, peroxy-nitrite, HOCl, chloramine) mostly contributed by neutrophils and macrophages.

Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Proanthocyanidins and other tannins are known to facilitate wound healing [7,8]. The mode of action, however, remained unclear. Grape seed proanthocyanidin extract has been reported to have various clinically relevant redox-active properties [9–14]. We have previously shown that oxidants potentially induce VEGF expression in human HaCaT keratinocytes [15, 16] and that a grape seed proanthocyanidin extract markedly potentiated oxidant-induced VEGF expression in human HaCaT keratinocytes [15]. The current study was undertaken to build on that previous finding and investigate the effects of the proanthocyanidin extract on dermal wound healing *in vivo*.

MATERIALS AND METHODS

Materials

A grape seed proanthocyanidin extract (GSPE; batch no. 005004 containing 5000 ppm trans-resveratrol) was obtained from InterHealth Nutraceuticals, Inc. (Benicia, CA, USA). GSPE is a natural extract containing approximately 54% dimeric, 13% trimeric, and 7% tetrameric proanthocyanidins, a small amount of monomeric bioflavonoids [12], and 5000 ppm of trans-resveratrol. This is the same extract used in our previous study testing the effects of GSPE on inducible VEGF expression *in vitro* [15]. Unless otherwise stated all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

Cells and cell culture

Immortalized human keratinocytes line HaCaT [17] were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu g/ml$ streptomycin.

GSPE stock solution for cell treatment was prepared

fresh in dimethyl sulfoxide at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1%. Respective controls were treated with equal volumes of dimethyl sulfoxide. HaCaT cells were pretreated with GSPE (2.5–10 $\mu g/ml$) for 24 h. Treatment of cells with GSPE did not influence cell viability as detected by a standard lactate dehydrogenase-dependent viability assay (not shown). Following incubation with GSPE, the cells were washed with serum-free medium and then treated with H_2O_2 (250 μM) in a serum-free medium.

Transient transfection and VEGF promoter-driven luciferase assay

HaCaT cells were transiently cotransfected with the VEGF-Luc and Renilla luciferase (pRL-SV40; Promega Corporation, Madison, WI, USA) constructs using FUGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions. VEGF-Luc represents a 2.6 kb VEGF promoter fragment (bp – 2361 to +298, relative to transcription start site) ligated to a luciferase reporter gene [18]. After 18–24 h of transfection, media was changed and GSPE was added to the cells. After 24 h, culture media was changed to serum-free media and cells were activated for 6 h with H_2O_2 . Dual luciferase reporter activity was determined using a commercial kit (Promega Corporation).

Secondary intention excisional dermal wound model

Male BalbC mice ($n = 9$) between 4 and 6 weeks of age were used. Anesthesia was induced by isoflurane inhalation. Two 16×8 mm full-thickness rectangular excisional wounds were placed on the dorsal skin, equidistant from the midline and adjacent to the four limbs [16]. These wounds were left to heal by secondary intention. In primary healing the wound is reapproximated (i.e., with sutures) immediately minimizing the tissue defect. In secondary healing the wound is left open and allowed to heal and contract without mechanical assistance. As a result there is (i) larger tissue defect, (ii) more reparative granulation tissue, (iii) a more intense inflammatory response, and (iv) increased wound contraction and collagen deposition. Wounded animals recovered in their cages and were fed mouse chow and water *ad libitum*. One of the two wounds were topically treated with 25 μl of 100 mg/ml GSPE suspended in saline (2.5 mg by weight) for a total of 5 consecutive days from the day of wounding (d 0). This dosage was determined on the basis of preliminary experiments directed at obtaining the minimum dose that caused a prominent differ-

ence in wound closure. For tissue collection, animals were killed by carbon dioxide narcosis on d 5 post-wounding and 1–1.5 mm of the wound edge was collected for histological and immunohistochemical studies. All animal protocols were approved by the Animal Institutional Lab Animal Care and Use Committee (ILACUC) of the Ohio State University, Columbus, OH.

Determination of wound area

Digital imaging of wounds was performed using a digital camera (Mavica FD91; Sony). The wound area was determined using WoundImager software. This software is a versatile tool for wound assessments and is designed to extrapolate physical measurements from a digital image [16].

Glutathione/glutathione disulfide ratio

Glutathione (GSH) and glutathione disulfide (GSSG) were simultaneously detected from wound-edge tissues using a high-performance liquid chromatography (HPLC)-coulometric electrode array detector (Coularray Detector, model 5600 with 12 channels; ESA Inc., Chelmsford, MA, USA). For extraction, wound-edge tissues were pulverized in liquid nitrogen and then homogenized in 5% wt/v *m*-phosphoric acid containing 0.1

mM Na₂EDTA using a Teflon homogenizer. The samples were snap-frozen and stored in liquid nitrogen until the HPLC assay. On the day of HPLC assay, samples were quickly thawed on ice and centrifuged (12,000 × *g*, 5 min) at 4° C. Supernatants were collected and filtered through a 0.22 μM filter. The filtrate was immediately injected to HPLC. GSH and GSSG were separated using a C₁₈ column and the following mobile phase: 50 mM sodium dihydrogen phosphate, 0.5 mM octanosulphonic acid, and 3% acetonitrile at pH 2.7 [19]. The coulometric electrode array offers several advantages over conventional single-channel detectors [20].

Histology

Formalin-fixed wound-edges were embedded in paraffin and sectioned. The sections (4 μm) were deparaffinized. All incubations and washes were carried out at room temperature (RT). Deparaffinized sections were washed three times in 0.05 M Tris-buffered saline and sections were treated with DAKO Target Retrieval solution. Endogenous peroxidase activity was blocked with 0.3% (v/v) H₂O₂ in TBS. The slides were washed three times with TBS and non-specific binding was blocked with 10% rabbit serum (VEGF) or 10% goat serum (for 4-HNE and tenascin) for 30 min. After three washes in TBS, the slides were

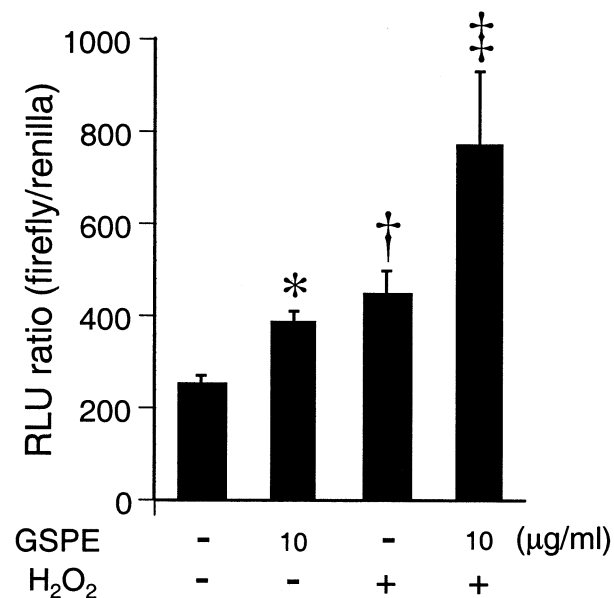


Fig. 1. Transcriptional control of inducible VEGF expression by GSPE. HaCaT cells were pretreated with GSPE (10 μg/ml) for 24 h. Cells were washed and treated with 250 μM H₂O₂ for 6 h. Mean ± SD. *Higher compared to GSPE nontreated cells for the effect of GSPE treatment alone; ‡higher compared to H₂O₂ non-treated cells for the effect of H₂O₂ alone; †higher compared to H₂O₂-treated cells for the effect of GSPE on inducible VEGF transcription. *p* < .05.

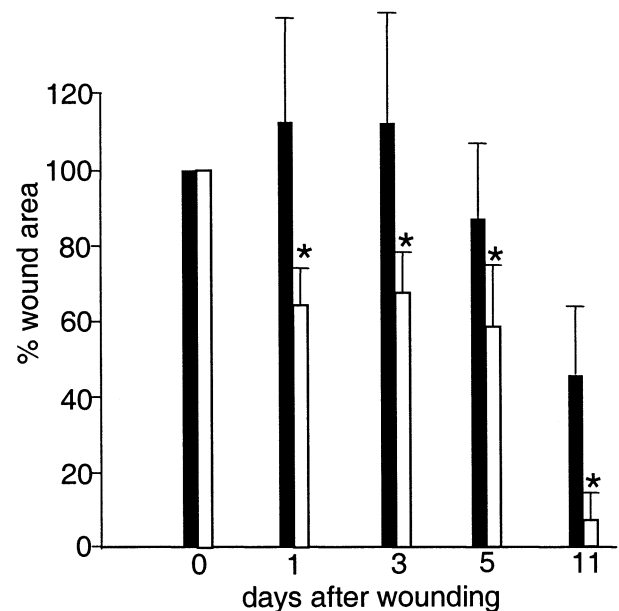


Fig. 2. Topical application of GSPE accelerated wound contraction and closure. Wound contraction shown as percentage of area of initial wound determined on the indicated day after wounding. Day 11 was the day when the first wound closed. *Lower in GSPE-treated (open bar) compared to corresponding placebo-treated (closed bar) wound area; *p* < .05.

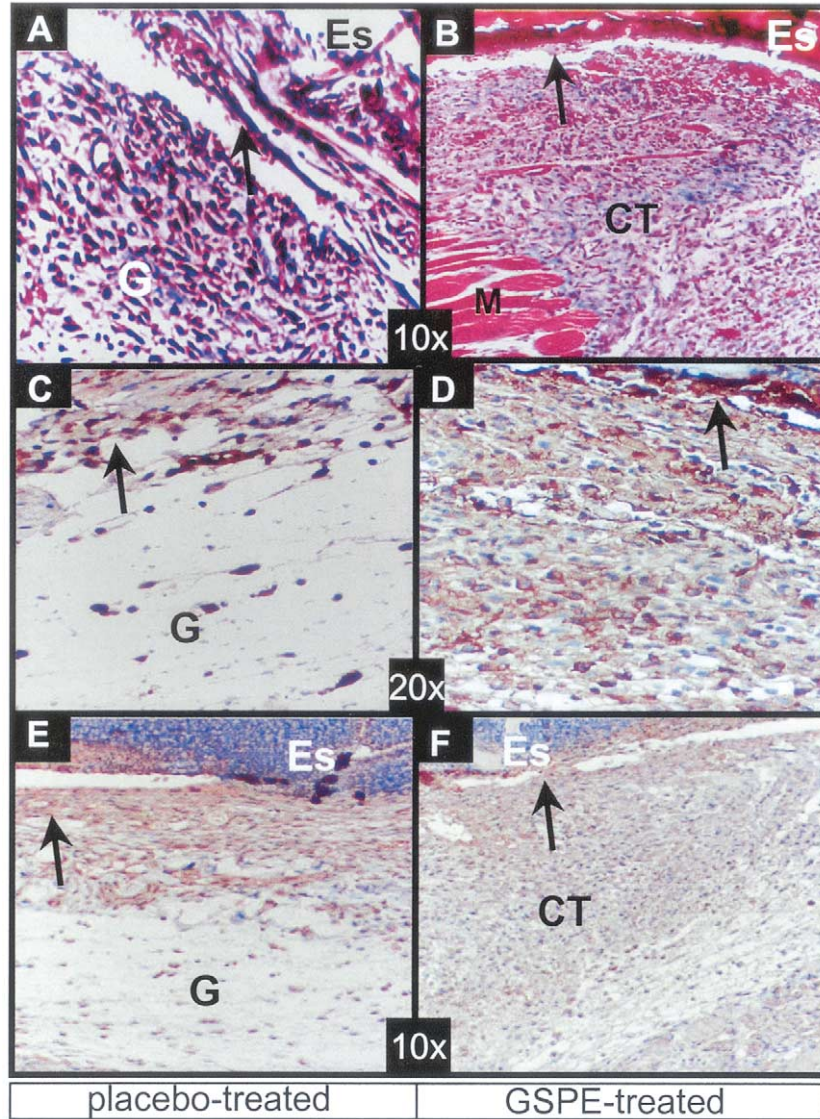


Fig. 3. Histological and immunohistochemical analysis of wound-edge tissue. Neutral buffered formalin-fixed paraffin sections were stained with Masson Trichrome (A,B), with anti 4-HNE (C,D), or anti-VEGF (E,F) antibodies. Trichrome staining procedure results in blue-black nuclei, blue collagen/cytoplasm, and keratin/muscle fibers/intracellular fibers all stained red. Note enhanced formation of epidermis and deposition of connective tissue in GSPE-treated tissue (B) compared to that in tissue from placebo-treated wound (A). Placebo-treated wound tissue was characterized with less granulation tissue (G) below hyperproliferative epithelium (HE) tissue. Cell density in granulation tissue was higher in GSPE-treated wounds. GSPE-treatment was associated with markedly higher presence of oxidant byproduct 4-hydroxynonenal (4-HNE) and VEGF. Magnification: 10 \times for (A–F); and 20 \times (C,D). Es = eschar tissue, CT = enhanced deposition of connective tissue, M = muscle tissue. Arrows indicate reepithelialization tissue.

incubated for 60 min with anti-VEGF (1:100 dilution; R& D Systems, Minneapolis, MN, USA; Fig. 3), anti-4-HNE (1:1000 dilution; Alexis Biochemical, San Diego, CA, USA; Fig. 3), or antitenascin C antibody (1:250 dilution, Chemicon International Inc, Temecula, CA, USA; Fig. 5). Next, the slides were washed with TBS and incubated with biotinylated secondary antibody for 30 min. This was followed by washing the slides with TBS and incubating them with streptavidin-horseradish peroxidase (HRP) complex

(DAKO LSAB + Kit, K090) for 15 min. After three washes, slides were incubated with substrate-chromogen solution (3,3-diaminobenzidine [DAB] from DAKO LSAB) for 5 min and counterstained with Mayer hematoxylin for 3 min [21]. For the staining of tenascin, streptavidin-HRP (Universal KIT; Vector Laboratories, Burlingame, CA, USA) and DAB (Sigma) solutions were used. The slides were then mounted with Gel Mount (Biomedica Corp., Foster City, CA, USA). Images were obtained using an Olympus

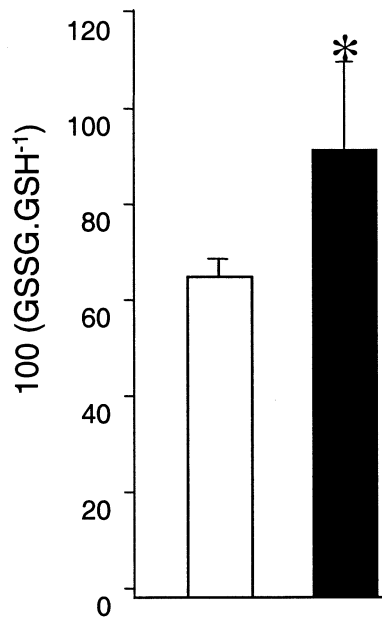


Fig. 4. Wound-edge glutathione redox status in response to GSPE treatment. Tissue samples were harvested d 5 post-wounding. Mean \pm SD. *Higher in tissue from GSPE-treated wound (closed bar) compared to that in tissues from placebo-treated wounds (open bar); $p < .05$.

MO 21 microscope fitted with Pixera digital camera and software.

Masson Trichrome staining. Wound-edge sections (4 μm) were deparaffinized and stained using Masson Trichrome procedure [22]. This procedure results in blue-black nuclei; blue collagen and cytoplasm; keratin, muscle fibers, and intracellular fibers all stained red.

Statistics

In vitro data are reported as mean \pm SD of at least three experiments. Comparisons among multiple groups were made by analysis of variance, $p < .05$ was considered statistically significant. In vivo data from one wound (placebo-treated control) of a mouse was compared to the other treated wound on the same mouse using paired *t*-test.

RESULTS

Previously we reported that GSPE potently upregulates oxidant-induced VEGF expression in human HaCaT keratinocytes. Pretreatment of cells with GSPE, increased H_2O_2 -induced VEGF protein as well as mRNA levels [15]. Increased mRNA levels may be a consequence of enhanced transcription and/or decreased degradation or increased stability of the specific mRNA. To

test whether GSPE indeed facilitates VEGF transcription, we conducted transactivation studies using a luciferase reporter construct driven by a VEGF promoter. The results demonstrate that GSPE pretreatment clearly facilitates H_2O_2 -induced VEGF transcription (Fig. 1). Using this analytical approach, it was also evident that GSPE alone is able to significantly ($p < .05$) turn on VEGF transcription (Fig. 1).

Based on in vitro results, we had previously hypothesized that GSPE would promote wound healing [15]. To test this hypothesis, male BalbC mice were used. Two 8×16 mm full-thickness excisional wounds were made on the dorsal skin, equidistant from the midline and adjacent to the four limbs as described previously [16]. These wounds were left to heal by secondary intention. To test the effect of GSPE on wound contraction and closure, each of the two wounds was topically treated either with saline or with 2.5 mg of GSPE for a total of 5 d from the day of wounding (d 0). Wound contraction and closure was studied by digital imaging followed by software-assisted analysis of wound area (WoundMatrix). Wound contraction and closure, shown as percentage of area of the initial wound, is demonstrated in Fig. 2. GSPE clearly ($p < .05$) accelerated wound closure and the effect was clearly seen from d 1 after wounding.

The wound edge (1–1.5 mm) was surgically removed and subjected to histological studies. For a more detailed visualization of the histological architecture of the wound-edge tissue, sections were subjected to trichrome staining as presented in Fig. 3. GSPE treatment was associated with enhanced formation of epidermis and deposition of connective tissue compared to that observed in the corresponding control wound on the same mouse. The placebo-treated wound edge was characterized by the presence of a less extensive granulation tissue below the reepithelialization tissue. In addition, the density of cells in the granulation tissue was clearly higher in the GSPE-treated wounds compared to that of the placebo-treated wounds (Fig. 3). GSPE-treated wounds were characterized by a more favorable histological organization (Fig. 3).

An oxidizing milieu in the wound microenvironment favors the expression of VEGF and supports the healing process [23]. In support of this we have previously demonstrated that oxidants such as H_2O_2 induces VEGF expression [15]. To test the effect of GSPE treatment on the oxidizing milieu in the wound microenvironment, wound-edges were stained for 4-hydroxynonenal (Fig. 3). In addition, glutathione redox state was determined from the same tissue samples (Fig. 4). Both markers consistently indicated that GSPE treatment was associated with enhanced markers of tissue oxidation at the wound site. Such enhanced footprints of oxidation in the

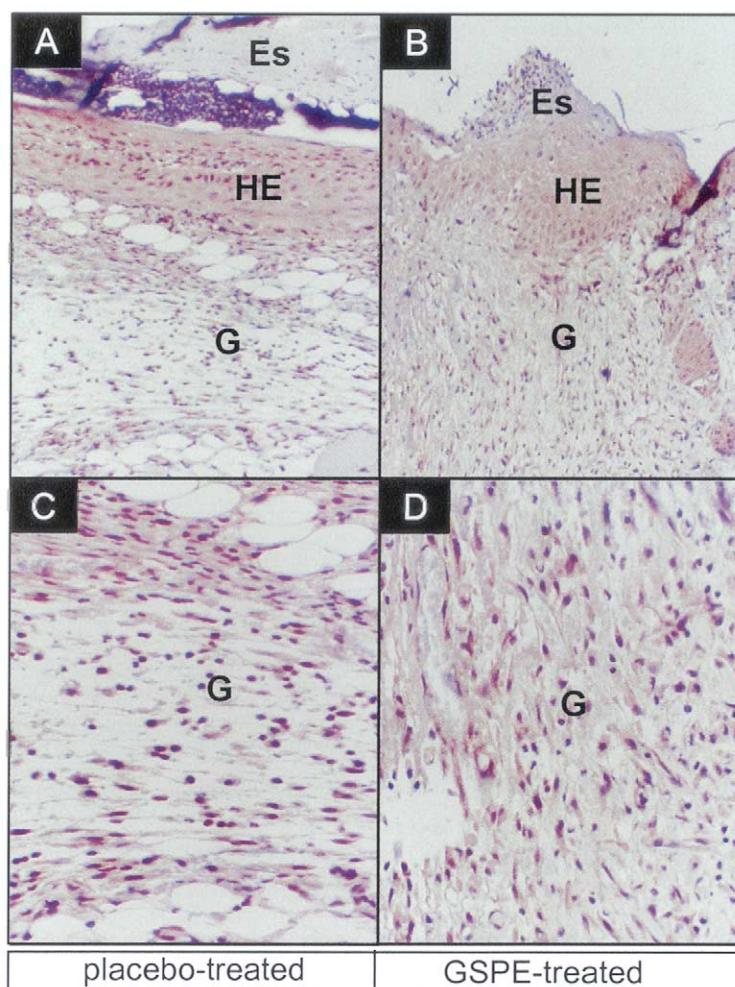


Fig. 5. Immunohistochemical localization of tenascin in wound-edge tissue. Tissue samples were harvested d 5 post-wounding. Increased presence of tenascin (brown stain) in the granulation tissue (G) region of GSPE-treated wounds compared to that of placebo-treated wounds. Es = eschar tissue, HE = hyperproliferative epithelium.

wound-edge tissue were associated with the increased presence of VEGF protein (Fig. 3).

Wound healing is known to be associated with rapid expression of tenascin [24,25]. Thus, expression of tenascin in the granulation tissue is often used as a marker of cutaneous wound repair [26]. We observed that while tenascin expression in the placebo-treated and GSPE-treated wounds were comparable in the hyperproliferative epithelial region, the expression of tenascin in the granulation tissue just below the hyperproliferative epithelium was clearly higher in GSPE-treated wound-edges (Fig. 5).

DISCUSSION

The transformation of digitalis from a folk medicine, foxglove, to a modern drug, digoxin, illustrates that the

study of complex natural extracts is a relevant approach to discover principles of modern pharmacology [27]. It is estimated that at present around 40% of the U.S. population uses a complementary and alternative medicine therapy in a given year [28]. Proanthocyanidins and other tannins are known to facilitate wound healing [7,8]. The mode of action, however, remains unclear. Previously, we have reported that GSPE upregulates inducible VEGF expression in human HaCaT keratinocytes [15]. Using a VEGF-luciferase reporter assay, we have now confirmed that GSPE upregulates inducible VEGF transcription. It was also evident that GSPE alone is capable of inducing VEGF transcription. These results present first evidence showing that a natural extract may have such specific effects on inducing and upregulating the transcription of an angiogenic gene.

Wound healing is a complex multifactorial process

that results in the contraction and closure of the wound and restoration of a functional barrier [29]. The restoration of a functional barrier is dependent on the successful regeneration of new skin with an architecture that closely resembles the injured tissue. While such regeneration is known to happen more often in the fetal tissue, adult tissue regeneration often suffers from a wide range of imperfections [30]. Topical GSPE markedly improved wound contraction and closure and the effects were distinctly visible from d 1 post-wounding. Masson staining of the wound edge provides evidence of a more desirable histological organization of the tissue in response to GSPE treatment. GSPE is known to enhance low-level production of intracellular NO [31], which in turn is known to facilitate VEGF expression in HaCaT keratinocytes [32]. Oxidants present at the wound site are thought to support wound repair [15,16,23]. GSPE treatment was associated with enhanced tissue oxidation at the wound site. While it is known that under certain conditions GSPE may demonstrate potent antioxidant properties [33], it must be considered that oxidative modification of antioxidants may result in the formation of potent oxidants. Importantly, such chemistry is possible at the wound site that is rich in oxidants delivered by phagocytic cells [23,34]. Tannins and tannic acid are an integral component of grape seed [35]. Tannic acid is known to be capable of generating hydroxyl radicals [36]. The radical structures obtained after oxidation of flavon(ol)s and proanthocyanidins have been characterized [37].

Extracellular matrix-receptor interactions have a profound influence on major cellular programs including growth, differentiation, migration, and survival. The tenascin family of glycoproteins display highly restricted and dynamic patterns of expression in the adult during normal processes such as wound healing, nerve regeneration, and tissue involution [38]. It has been reported recently that reactive oxygen species may serve as a signal to trigger tenascin expression [39]. It is therefore plausible that enhanced oxidant status in GSPE-treated wounds was responsible for the observed increase in tenascin expression.

In summary, our current study provides firm evidence to support that topical application of GSPE represents a feasible and productive approach to support dermal wound healing. Taken together, this and the preceding report of in vitro findings [15] indicate that GSPE is capable of inducing and upregulating inducible VEGF transcription. Topical GSPE markedly enhances wound contraction and improves histological reorganization of the regenerating tissue.

Acknowledgements — Supported by National Institutes of Health GM 27345 to C.K.S. The Laboratory of Molecular Medicine is the research

division of the Center for Minimally Invasive Surgery. We thank Jelena Vider, M.D. for technical assistance.

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