Free Radical Biology & Medicine, Vol. 31, No. 1, pp. 38–42, 2001

0891-5849/01/$–see front matter

PII S0891-5849(01)00544-5

Original Contribution

UPREGULATION OF OXIDANT-INDUCED VEGF EXPRESSION IN CULTURED KERATINOCYTES BY A GRAPE SEED PROANTHOCYANIDIN EXTRACT

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(*Received* 13 *October* 2000; *Accepted* 21 *March* 2001)

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. Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. This study provides first evidence showing that natural extracts such as grape seed proanthocyanidin extract containing 5000 ppm resveratrol (GSPE) facilitates oxidant-induced VEGF expression in keratinocytes. Using a ribonuclease protection assay (RPA), the ability of GSPE to regulate oxidant-induced changes in several angiogenesis-related genes were studied. While mRNA responses were studied using RPA, VEGF protein release from cells to the culture medium was studied using ELISA. Pretreatment of HaCaT keratinocytes with GSPE upregulated both hydrogen peroxide as well as TNF-α-induced VEGF expression and release. The current results suggest that GSPE may have beneficial therapeutic effects in promoting dermal wound healing and other related skin disorders. © 2001 Elsevier Science Inc.

Keywords—Wound healing, Angiogenesis, Redox, Tannins, Proanthocyanidins, Free radicals

INTRODUCTION

Angiogenesis plays a central role in wound healing. Among many known growth factors, VEGF is believed to be the most prevalent, efficacious, and long-term signal that is known to stimulate angiogenesis in wounds [1]. VEGF is a homodimeric glycoprotein that is highly conserved and shares structural homology with placental 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. These effects of VEGF are mediated through two distinct high-affinity endothelial cell receptors, flt-1 [5,6] and KDR/Flk-1 [7,8], having protein-tyrosine kinase domains [9].

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 [10]. This reactive oxygen species 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. 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.

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, $O_2^{\bullet -}$, $^{\bullet}NO$, peroxynitrite, HOCl, chloramine) mostly contributed by neutrophils and macrophages. In a variety of cell types, these oxidants are known to markedly increase expression and release of VEGF [11–13]. Proanthocyanidins or

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condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Grape seed proanthocyanidin extract has been reported to have various clinically relevant redox-active properties [14–18]. This study provides first evidence showing that natural extracts derived from grape seeds may facilitate oxidant-induced VEGF expression in keratinocytes. Current results suggest that grape seed-derived natural extracts may have beneficial effects in promoting dermal wound healing and other related skin pathologies.

MATERIALS AND METHODS

Materials

A novel IH636 grape seed proanthocyanidin extract (commercially known as ActiVin, batch # 005004 containing 5000 ppm trans-resveratrol, GSPE) 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 [19], and 5000 ppm of trans-resveratrol. 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 [20] 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 μg/ml streptomycin.

GSPE stock solution for cell treatment were 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 volume of dimethyl sulfoxide. HaCaT cells were pretreated with GSPE (2.5–15 μg/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.

RNAse protection assay

The mRNA were detected in total cell RNA by RNAase protection assay (RPA) utilizing DNA templates from BD Pharmingen (BD RiboQuant Ribonuclease Protection Assay System; BD PharMingen, San Diego, CA, USA). The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection of mRNA species. The assay is based on the characterization of DNA-dependent RNA polymerases from the bacteriophages SP6, T7, and T3, and the elucidation of their cognate promoter sequences. These polymerases are ideal for the synthesis of high-specific-activity RNA probes from DNA templates because these polymerases exhibit a high degree of fidelity for their promoters, polymerize RNA at a very high rate, efficiently transcribe long segments, and do not require high concentrations of rNTPs. Thus, a cDNA fragment of interest can be subcloned into a plasmid that contains bacteriophage promoters, and the construct can then be used as a template for synthesis or radiolabeled antisense RNA probes. Two distinct advantages of the multiprobe RPA approach are its sensitivity and its capacity to simultaneously quantify several mRNA species in a single sample of total RNA. This allows comparative analysis of different mRNA species within samples, and, by incorporating probes for housekeeping gene transcripts, the levels of individual mRNA species can be compared between samples.

Radiolabeled, single-strand cRNA of the indicated genes was synthesized at room temperature utilizing Redivue uridine 5′-[α-^{32}P]triphosphate, triethylammonium salt (3000 Ci/mmol; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) and T7 polymerase. RNA samples (2–10 μg), including murine control RNA and yeast tRNA (2 μg) as positive and negative controls, were dried and then resuspended in 8 μl hybridization buffer and 2 μl of radiolabeled probe ($\sim 3 \times 10^5$ cpm/μl). The hybridization mixtures were overlaid with mineral oil, denatured at 90°C, and incubated for 16–18 h in a prewarmed 56°C oven. After incubation, single-strand RNA was digested in an RNase A/T1 cocktail, followed by proteinase K digestion. The remaining radiolabeled RNA fragments, protected from degradation by hybridization to homologous cellular mRNA, were electrophoresed on a 6% acrylamide-urea gel with a radiolabeled probe set ($1–2 \times 10^3$ cpm) as size markers. The gels were dried and analyzed by phosphorimaging. The data were normalized to ribosome-associated protein L32 (L32) or GAPDH mRNA content as shown in Fig. 1.

Measurement of VEGF protein

HaCaT cells were seeded onto multiple-well culture plates. After 24 h of growth, cells were treated with GSPE for 24 h. After this duration, treated cells were washed with serum-free medium and then challenged with H_2O_2 or TNF-α in serum-free medium for 12 h. The reason for selecting serum-free medium was to avoid any

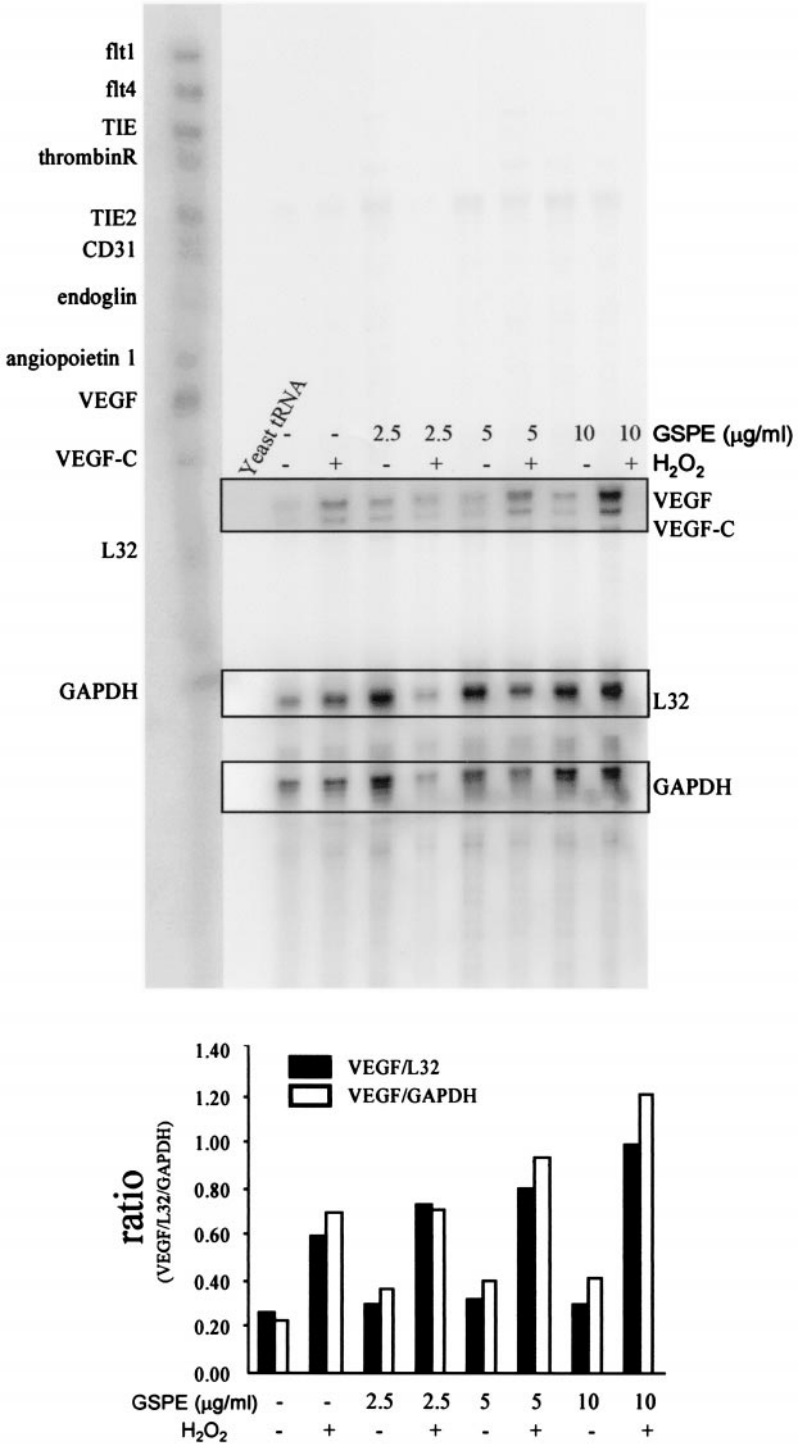

Fig. 1. GSPE upregulates oxidant-induced VEGF mRNA expression. HaCaT cells were pretreated with GSPE (2.5–10 μg/ml) for 24 h. Following incubation the cells were washed with serum-free medium and then activated with H_2O_2 (250 μM) in a serum-free medium for the next 6 h. Total RNA was extracted after 6 h and VEGF and VEGF-C mRNA expression was assessed by ribonuclease protection assay (RPA) (upper panel). Yeast tRNA was used as negative control. The gels were dried and analyzed by phosphorimaging. The data were normalized to ribosome-associated protein L32 (L32) or GAPDH mRNA content (lower panel). Representative of three independent experiments. Flt1 and Flt4 = receptor tyrosine kinases, to which VEGF binds on endothelial cells; TIE and TIE2 = tyrosine kinase receptors, also known as Tek; CD31 = also known as PECAM-1; VEGF = vascular endothelial growth factor.

possible interaction between the serum components and H_2O_2. VEGF level in the medium was determined using commercially available ELISA kit (R & D Systems, Minneapolis, MN, USA).

RESULTS AND DISCUSSION

The RPA approach to detect mRNA is highly specific and quantitative due to the RNase sensitivity of mismatched base pairs and the use of solution-phase hybridization driven toward completion by excess probe. HaCaT cells were observed to be highly sensitive to hydrogen peroxide-induced VEGF expression (Figs. 1 and 2). Previous report using this cell type showed that 1 mM hydrogen peroxide induces VEGF expression [11]. Our results consistently showed that 0.25 mM hydrogen peroxide significantly increased the expression of VEGF. Oxidant-induced VEGF expression was detected both at the level of mRNA as well as protein. Using the ribonuclease protection assay, we studied the response of multiple angiogenesis-related genes to oxidant treatment (Fig. 1). No other gene studied was sensitive to oxidant treatment, suggesting that the transcriptional control of VEGF gene is specifically sensitive to hydrogen peroxide. The ability of oxidants such as hydrogen peroxide to induce the expression and release of VEGF has been confirmed by in vivo studies as well [12,13]. Among many known growth factors, VEGF is believed to be the most prevalent, efficacious, and long-term signal that is known to stimulate angiogenesis in wounds [1]. Therefore strategies to sensitize cells to oxidant-induced VEGF expression and release should facilitate dermal wound healing.

Proanthocyanidins and other tannins are known to facilitate wound healing [21,22]. The mode of action, however, remains unclear. In this study, we observed that keratinocytes pretreated with GSPE were sensitized to oxidant-induced VEGF mRNA (Fig. 1) expression as well as VEGF protein release from the cells to the culture medium (Fig. 2). To avoid direct interaction of GSPE with hydrogen peroxide, cells were washed with GSPE-free medium before oxidant treatment. The results obtained suggest that GSPE pretreatment influenced the transcription regulatory processes that regulate oxidant-induced VEGF expression. The ability of GSPE to influence inducible VEGF expression and release was also tested in response to treatment of cells by another major wound-related cytokine, TNF-α. Consistent with findings using hydrogen peroxide, GSPE treatment upregulated TNF-α-induced VEGF mRNA expression (not shown) as well as VEGF protein release (Fig. 2). We have recently shown potent inhibitory effect of low concentrations of GSPE on cytokine-induced expression of

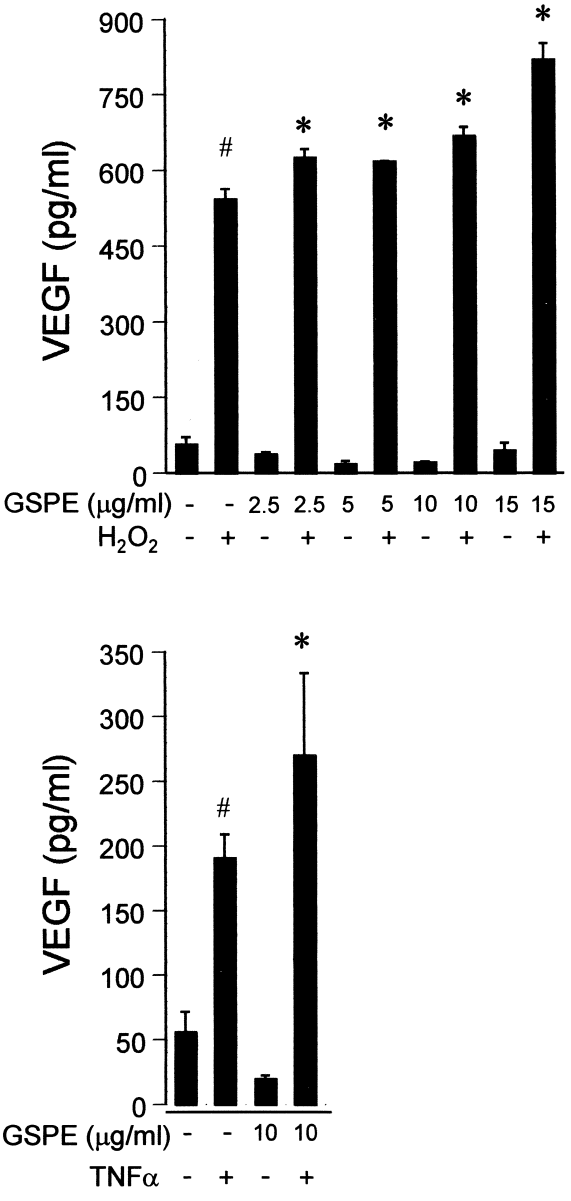

Fig. 2. GSPE pretreatment increases oxidant as well as TNF-α-induced VEGF protein release. HaCaT cells were seeded onto multiple-well culture plates. After 24 h of growth, cells were treated with GSPE (2.5–15 μg/ml) for 24 h. After this duration, treated cells were washed with serum-free medium and then challenged with 250 μM H_2O_2 (upper panel) or 20 ng/ml TNF-α for 12 h (lower panel). VEGF level in the medium was determined using commercially available ELISA kit. #Significantly ($p < .05$) higher than corresponding H_2O_2 or TNF-α-nontreated cells. *Significantly ($p < .05$) higher than corresponding H_2O_2 or TNF-α-treated cells.

adhesion molecules. Adhesion molecules play a major role in the regulation of inflammatory processes [23].

At present, the signaling pathways involved in oxidant-induced VEGF expression are not known and further studies are necessary to determine the mechanisms by which GSPE regulate such processes. The current

findings, however, do provide the first evidence supporting the historical belief that proanthocyanidins or condensed tannins promote wound healing [21,22]. GSPE may have a beneficial role in regulating wound healing and other related skin disorders. Results presented in this study establish that GSPE regulates transcriptional control of inducible VEGF expression by modulating pathways that are common to both H_2O_2 as well as TNF-α signaling. Further studies examining the cellular and molecular basis of wound healing regulation by natural proanthocyanidins is warranted.

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