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Copper-induced vascular endothelial growth factor expression and wound healing

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Sen, Chandan K., Savita Khanna, Mika Venojarvi, Prashant Trikha, E. Christopher Ellison, Thomas K. Hunt, and Sashwati Roy. Copper-induced vascular endothelial growth factor expression and wound healing. *Am J Physiol Heart Circ Physiol* 282: H1821–H1827, 2002. First published January 17, 2002; 10.1152/ajpheart.01015.2001.—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. Whereas a direct role of copper to facilitate angiogenesis has been evident two decades ago, the specific targets of copper action remained unclear. This report presents first evidence showing that inducible VEGF expression is sensitive to copper and that the angiogenic potential of copper may be harnessed to accelerate dermal wound contraction and closure. At physiologically relevant concentrations, copper sulfate induced VEGF expression in primary as well as transformed human keratinocytes. Copper shared some of the pathways utilized by hypoxia to regulate VEGF expression. Topical copper sulfate accelerated closure of excisional murine dermal wound allowed to heal by secondary intention. Copper-sensitive pathways regulate key mediators of wound healing such as angiogenesis and extracellular matrix remodeling. Copper-based therapeutics represents a feasible approach to promote dermal wound healing.

redox; oxidant; skin; angiogenesis; repair

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 (28). VEGF is a homodimeric glycoprotein that is highly conserved and shares structural homology with placenta growth factor and platelet-derived growth factor (24, 39). It induces migration and proliferation of endothelial cells and enhances vascular permeability (19) consistent with the purported ability to promote angiogenesis. These effects of VEGF are mediated through two distinct high-affinity endothelial cell receptors, flt-1 (10, 33) and KDR/Flk-1 (31, 38), having protein-tyrosine kinase domains (27). 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 $O_2^-\cdot$, which is very well known to be critical for defense against bacteria and other pathogens (2). This reactive oxygen species may be rapidly converted to a variety of species such as H_2O_2, ·OH or $ONOO^-$, depending on the local conditions. The wound site is rich in both oxygen- and nitrogen-centered reactive species along with their derivatives mostly contributed by neutrophils and macrophages. Previously, we have reported that H_2O_2 induces VEGF expression in human keratinocytes (21). It has been hypothesized that wound-related oxidants support wound repair by promoting angiogenesis (34). ·OH is a stronger oxidizing species than H_2O_2. We sought to determine whether inducible VEGF expression is sensitive to the strength of reactive oxygen species. Human keratinocytes were exposed to H_2O_2 or ·OH, and expression of VEGF was studied. ·OH was generated using the Haber-Weiss reaction strategy reacting H_2O_2 and Fe^{2+} or Cu^{2+} as transition metal-ion catalysts. These two metal ions have been identified as potential mediators of ·OH generation in biological tissues (17). In particular, the wound site is rich (~30 μM) in copper (16).

MATERIALS AND METHODS

Materials. Unless otherwise stated all other chemicals and reagents were obtained from Sigma (St. Louis, MO) and were of analytic grade or the highest grade available.

Cells and cell culture. Immortalized human keratinocytes line HaCaT (7) were grown in Dulbecco's modified Eagle's medium (Life Technologies; Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C (21). Adult human epidermal keratinocytes (HEKa) were obtained from Cascade Biologics (Portland, OR). HEKa were cultured in the EpiLife medium supplemented with EpiLife Defined Growth Supplement supplied by the Cascade Biologics. The cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C.

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Reverse transcription-polymerase chain reaction. HaCaT cells were grown in 100-mm × 20-mm culture plates to confluence and rendered quiescent by changing to serum-free DMEM for 12 h. After 12 h cells were challenged with 25 μM copper sulfate for 6 h, and total RNA was extracted using the TRIzol reagent (GIBCO-BRL) according to the manufacturer's instruction. RT was performed using an RNA PCR Kit (Perkin Elmer). For RT-PCR, 8 μg of total RNA was reverse transcribed to cDNA following the manufacturer's procedure. RT-generated cDNA encoding human VEGF and β-actin genes were amplified by PCR using specific primers (VEGF, R&D Systems; Minneapolis, MN) and β-actin (Stratagene; La Jolla, CA) as described below. The reaction volume was 50 μl containing (final concentration) PCR buffer (×1), deoxynucleotide (0.2 mM each), $MgCl_2$ (1.5 mM), Taq DNA polymerase (2.5 U), oligonucleotide primers (7.5 μM each), and RT products. After an initial denaturation for 2 min at 95°C, 30 cycles of amplification (94°C for 45 s, 65°C for 45 s, and 72°C for 45 s) were performed followed by a 10-min extension at 72°C. An aliquot (10 μl) from each PCR reaction was electrophoresed in a 2% agarose gel containing 0.25 μg/ml ethidium bromide. The gel was then photographed under conditions of ultraviolet transillumination. For quantification, the VEGF signals were normalized relative to the corresponding β-actin signal from the same sample using the NIH Image 1.58b29 software.

VEGF protein assay. Cells were seeded onto multiple-well culture plates. After 24 h of growth (at ~80% confluency), the cells were synchronized by culturing in serum-deprived medium for 12 h. After the synchronization, cells were treated for 12 h with $CuSO_4$, H_2O_2, or other compounds as indicated in the respective figure legends. VEGF protein level in the medium was determined using a commercially available ELISA kit (R&D systems).

Secondary intention excisional dermal wound model. Male BalbC mice ($n = 10$) between 4 and 6 wk of age were used. Anesthesia was induced by isofluorane inhalation. Two 16-mm × 8-mm full-thickness rectangular excisional wounds were placed on the dorsal skin, equidistant from the midline and adjacent to the four limbs (see Fig. 3). These wounds were left to heal by secondary intention. 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 25 μM $CuSO_4$ (0.625 nmoles by weight) for 6 consecutive days from the day of wounding (*day 0*). For tissue collection, animals were killed by carbon dioxide narcosis on the fifth day postwounding, and 1–1.5 mm of the wound edge was collected for histological and immunohistochemical studies. All animal protocols were approved by 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.

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. Deparaffinized section were washed three times in 0.05M Tris-buffered saline (Dako TBS, Code S3001), and sections were treated with Dako Target Retrieval solution. Endogenous peroxidase activity was blocked with 0.3% (vol/vol) H_2O_2 in TBS. The slides were washed three times with TBS, and nonspecific binding was blocked with 10% rabbit serum for 30 min. After three washes in TBS, the slides were incubated for 60 min with anti-VEGF (1:100 dilution; R&D Systems, Minneapolis, MN) antibody (see Fig. 4). 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 complex (Dako LSAB + Kit, K090) for 15 min. After three washes, the slides were incubated with substrate-choromogen solution (3,3-diaminobenzidine, DAB from Dako) for 5 min and counterstained with Mayer hematoxylin for 3 min (12). The slides were then mounted with Rapid Mount (Histology Control System). Images were obtained using an Olympus MO 21 microscope fitted with Pixera digital camera and software. Wound-edge sections (4 μm) were deparaffinized and stained using Masson Trichrome procedure (3). This procedure results in blue-black nuclei; blue collagen and cytoplasm; and red-stained keratin, muscle fibers, and intracellular fibers.

Statistics. In vitro data are reported as means ± SD of at least three experiments. Comparisons among multiple groups were made by analysis of variance ANOVA. $P < 0.05$ was considered statistically significant. In vivo, data from one wound (placebo-treated control) of a mouse were compared with the other treated wound on the same mice using paired *t*-test.

RESULTS

To compare the relative efficacy of H_2O_2 and ·OH in inducing VEGF expression, HaCaT keratinocytes were treated with H_2O_2 alone or in combination with $FeSO_4$ or $CuSO_4$ (20, 42). The level of VEGF protein expressed in response to H_2O_2 and $FeSO_4$ was similar to that induced by H_2O_2 alone (Fig. 1*A*). However, HaCaT cells exposed to a combination of H_2O_2 and $CuSO_4$ expressed significantly higher levels of VEGF protein compared with that expressed in response to H_2O_2 treatment alone (Fig. 1*B*). Control experiments performed to test the individual effects of $FeSO_4$ and $CuSO_4$ on inducible VEGF expression by HaCaT cells revealed that, whereas VEGF expression is not sensitive to $FeSO_4$ (not shown), $CuSO_4$ alone potently induces VEGF expression in both HaCaT as well as human primary HEKa keratinocytes (Fig. 1, *C* and *D*). Given that 30 μM copper is expected to present in a wound site (16), the concentration of $CuSO_4$ required to induce marked VEGF expression was well within a relevant range. At 10 μM, $CuSO_4$ resulted in over 2.5-fold increase in VEGF expression compared with resting cells in standard culture. Further increase in the concentration of $CuSO_4$ treatment to 25 and 50 μM significantly increased VEGF expression. RT-PCR analysis revealed that $CuSO_4$ potently induced the 165-splice variant form of VEGF and that $CuSO_4$ regulates VEGF transcription (Fig. 2*D*).

Several studies have shown that oxidant-insult of cells may result in breakdown of intracellular proteins and release of transition metal ions such as copper (40). Thus we examined whether intracellular copper plays a role in H_2O_2-induced VEGF expression. Indeed, H_2O_2-induced VEGF expression was inhibited in cells treated with the Cu^{2+} chelator, diethylenetriaminepentaacetic acid (DTPA; Fig. 2*A*). Protocatechuic acid (3,4-dihydroxybenzoic acid) is a copper chelator that is used for the treatment of copper-overload Wilson's dis-

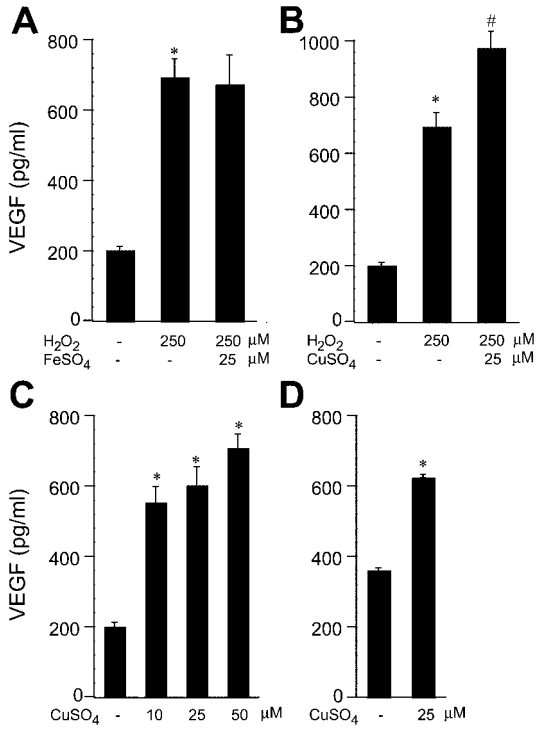

Fig. 1. Vascular endothelial growth factor (VEGF) protein expression in response to H_2O_2 and $FeSO_4$ (*A*), H_2O_2, and $CuSO_4$ (*B*) in human HaCaT keratinocytes. Dose-dependent Cu-induced VEGF expression in HaCaT (*C*) and primary human HEKa keratinocytes (*D*). Data are means ± SD. * and #$P < 0.05$: *higher in response to H_2O_2 or $CuSO_4$ treatment; #higher compared with cells treated with H_2O_2.

ease (23). Consistent with our observation with DTPA (Fig. 2*A*), protocatechuic acid treatment inhibited H_2O_2-induced VEGF expression, suggesting that copper may play a role in mediating the stimulatory effect of H_2O_2 on inducible VEGF expression (Fig. 2*B*). In some cell types, hypoxia induces VEGF expression by the presence of cytosolic c-Src kinase upstream and by hypoxia-inducible factor-1 (HIF1) downstream (8, 26). We sought to examine whether protein tyrosine kinases are involved in $CuSO_4$-induced VEGF expression. Inhibitors of protein tyrosine kinases clearly abrogated $CuSO_4$-induced VEGF protein as well as mRNA expression (Fig. 2, *C* and *D*).

In addition to the Fenton and Haber-Weiss type reactions, Cu^{2+} augment oxidative stress by a variety of other mechanisms such as metal-catalyzed oxidation (36) and thiol oxidation (37). Cu^{2+} can result in the formation of H_2O_2 during oxidation of homocysteine (37). To test whether oxidants were involved in $CuSO_4$-induced VEGF expression, an antioxidant-defense system of cells was impaired by arresting GSH synthesis using L-buthionine-*S*-*R*-sulfoximine. $CuSO_4$-induced VEGF expression was potentiated in GSH-deficient cells, indicating the possible involvement of redox-active events and an inhibitory role of cellular GSH (Fig. 2*E*). Given that inducible VEGF expression is known to be oxidant sensitive in these cells (21), it is appropriate to question whether Cu^{2+}-induced VEGF expression is directly dependent on H_2O_2. Our observation that Cu^{2+} chelators efficiently inhibited H_2O_2-induced VEGF expression indicates that Cu^{2+} acts independent of H_2O_2 (Fig. 2, *A* and *B*). Taken together, our results indicate that the effect of Cu^{2+} on inducible VEGF expression is not mediated by H_2O_2 but is dependent on the thiol-redox state of the cells.

The redox regulation aspect of mitogen-activated protein kinase (MAPK) has not been so well studied as protein tyrosine kinases. However, it has been shown that these serine-threonine kinases may be activated by H_2O_2 (15). We have studied the effect of three MAPK inhibitors: SB-203580, a highly specific inhibitor of MAPK; SB-202190, a potent inhibitor of p38 MAPK; and PD-98059, a selective inhibitor of MAPK kinase (MAPKK) or MEK. All three inhibitors were able to potently inhibit $CuSO_4$-induced VEGF expression (Fig. 2*F*). Phosphatidylinositol 3-kinase (PI3K) is redox sensitive. It may be activated by oxidants directly (11) or by oxidant-sensitive tyrosine kinases (30). Thus we tested the effect of wortmannin, a potent and selective PI3K inhibitor on VEGF expression induced by $CuSO_4$. Figure 2*G* shows that $CuSO_4$-induced VEGF expression was inhibited in the presence of wortmannin, suggesting that PI3K may be involved in the signaling path. The observation that each of the signal transduction inhibitors used in the current study completely abolished the ability of $CuSO_4$ to induce VEGF expression suggests that these kinases function in series to constitute a signaling path that is critical for VEGF expression. Indeed it is known that MAPK and c-Src are components of one signal transduction cascade (9). Importantly, the MAPK cascade, c-Src, and PI3K are all known to be responsible for the activation of HIF-1, a key regulator of inducible VEGF expression (6, 13, 35).

To further assess the role of copper in wound contraction and closure, male BalbC mice were used. Two 8-mm × 16-mm full-thickness excisional wounds were made on the dorsal skin, equidistant from the midline (Fig. 3, *inset*). These wounds were left to heal by secondary intention. To test the effect of copper on wound contraction and closure, each of the two wounds was topically treated with either placebo sterile saline or with the 25 μl of 25 μM $CuSO_4$ once a day for 5 days. On *day 5*, the wound edge (1–1.5 mm) was surgically removed and subjected to immunohistochemical studies. Wounds were digitally imaged and the wound area was estimated using a specialized WoundImager software. Topical treatment of the wound site with $CuSO_4$ clearly accelerated wound contraction and closures (Fig. 3). Histological analysis of wound-edge tissue substantiated that $CuSO_4$ treatment did not only accelerate wound closure but that the quality of regenerating tissue was distinctly different. $CuSO_4$ treatment was associated with more hyperproliferative epithelial tissue, and the density of cells in the granulation layer of copper-treated wounds was clearly higher (Figs. 4, *A1–A3*). Immunohistochemical evidence showing that wound edges of copper-treated wounds had more prominent VEGF expression is presented in Fig. 4*B*.

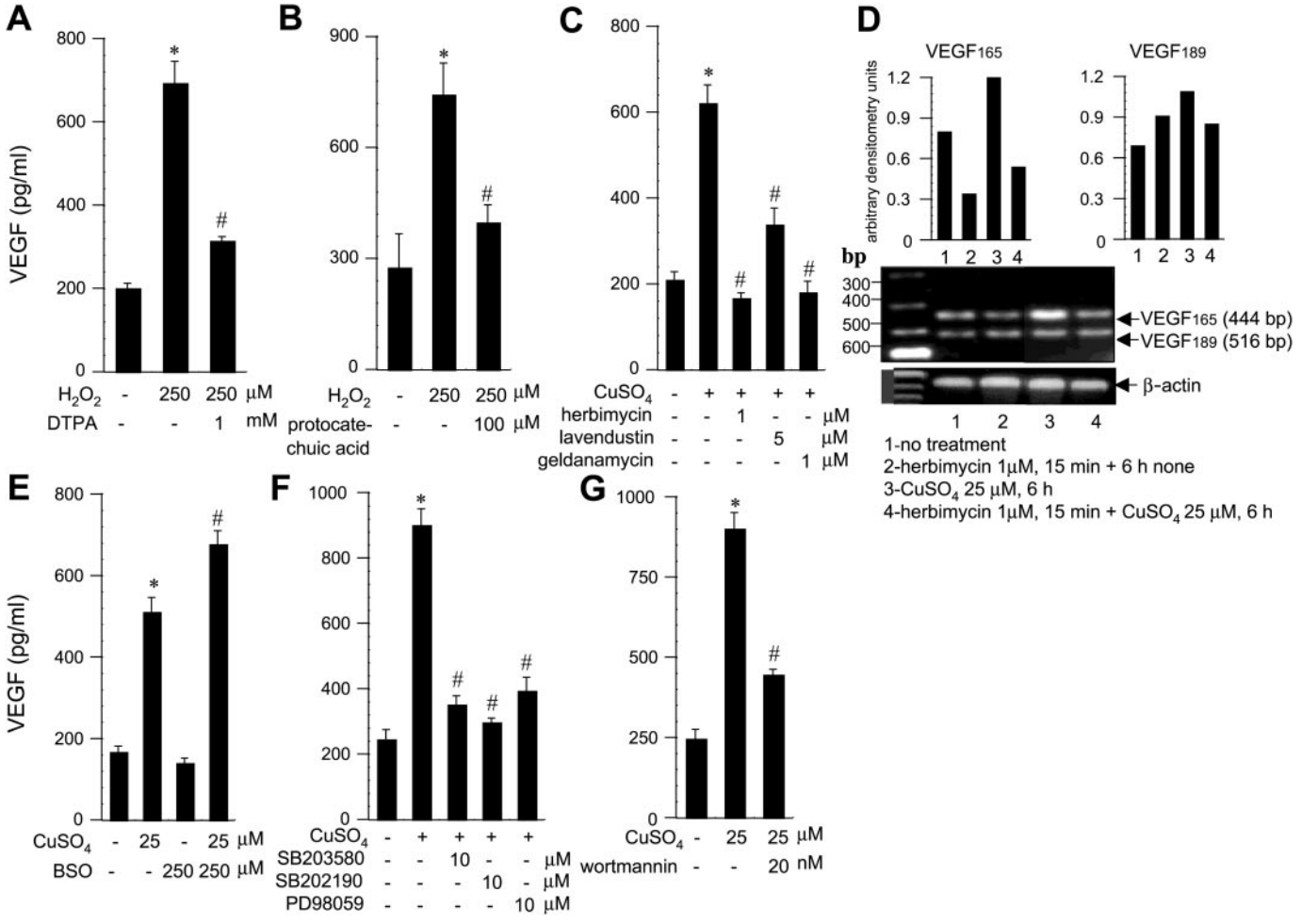

Fig. 2. *A*: Cu^{2+} chelator diethylenetriaminepentaacetic acid (DTPA) inhibited H_2O_2-induced VEGF expression by HaCaT keratinocytes. *B*: Cu^{2+} binding agent protocatechuic acid inhibited H_2O_2-induced VEGF expression. Role of protein tyrosine kinase inhibitors in Cu-induced VEGF protein (*C*) and mRNA expression (*D*). *E*: Cu-induced VEGF expression in *L*-buthionine-*S*-*R*-sulfoximine (BSO)-treated GSH-deficient cells. Regulation of Cu-induced VEGF expression by mitogen-activated protein kinase (MAPK) (*F*) and phosphatidylinositol 3-kinase (PI3K) (*G*) inhibitors. Data are means ± SD. * and #$P < 0.05$: *higher in response to H_2O_2 or $CuSO_4$ treatment; #significantly different from cells treated with H_2O_2 or $CuSO_4$.

DISCUSSION

A study that was designed to compare the VEGF-inducing properties of H_2O_2 and ·OH culminated in elucidating a major mechanism responsible for the angiogenic properties of copper. Whereas a direct role of copper to facilitate angiogenesis has been evident since two decades ago (32), the specific targets of copper action remained unclear. In a recent study, categorical effort was made to identify the cytokine(s) responsible for mediating the angiogenic effects of copper (18). The study reported that copper-induced proliferation of endothelial cells is not inhibited by serum deprivation or by the presence of antibodies against a variety of angiogenic, growth, and chemotactic factors, including angiogenin, fibroblast growth factors, epidermal growth factor, platelet-derived growth factor, tumor necrosis factor-α, transforming growth factor-β, macrophage/monocyte chemotactic and activating factor, and macrophage inflammatory protein-1α. Ironi-

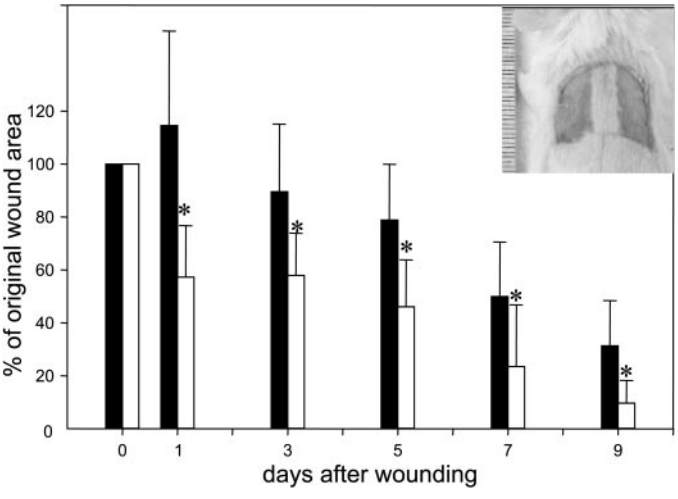

Fig. 3. The 8- × 16-mm full-thickness excisional wound model (*inset*). Wound contraction and closure in response to 0.625 nmoles of $CuSO_4$ treatment. Solid bar, placebo-treated control wound; Open bar, $CuSO_4$-treated wound. Data are means ± SD. *$P < 0.05$.

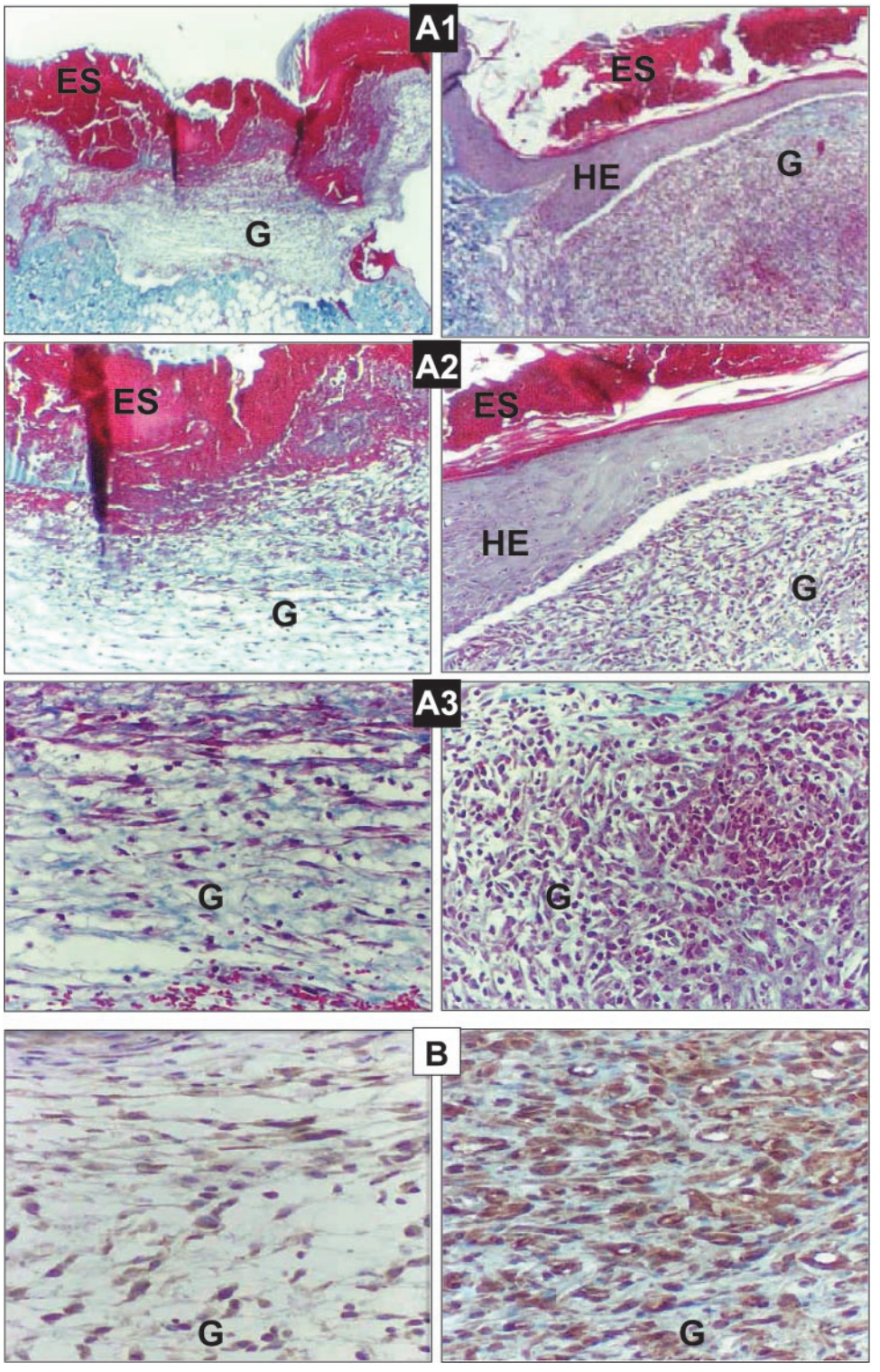

Fig. 4. Histological and immunohistochemical analysis of wound-edge tissue harvested 5 days after wounding. *A1–A3*: Masson Trichrome staining (*A1*, ×4; *A2*, ×10; *A3*, ×20). *B*: VEGF (×20). *Left*: placebo-treated control; *right*: $CuSO_4$ treated once a day for 5 days after wounding. Note that wound edge of $CuSO_4$-treated mice is characterized by the enhanced formation of hyperproliferative epidermis (HE), deposition of connective tissue, and a more tight arrangement of cells in the granulation layer (G). VEGF expression is clearly more prominent in tissue from Cu-treated mice compared with that from placebo-treated mice. ES, Eschar tissue.

cally, VEGF was not studied (18). In both in vitro (1) as well as in vivo (29) models, $CuSO_4$ clearly promote angiogenic responses. These observations have led to the development of anti-copper-based, anti-angiogenic strategies for the treatment of cancer (41). This report presents first evidence showing that inducible VEGF expression is sensitive to copper and that the angiogenic potential of copper may be harnessed to accelerate dermal wound healing.

In studies related to hypoxia-induced VEGF expression it was identified that c-Src tyrosine kinase plays a central role in regulating VEGF transcription (26). Ras-mediated activation of p42/p44 MAP kinases is known to exert a prominent action on inducible VEGF

expression at the transcriptional level. In normoxic conditions, p42/p44 MAPKs activate the VEGF promoter at the proximal (−88/−66) region where Sp-1/AP-2 transcriptional factor complexes are recruited. Under conditions of hypoxia, the stabilized and nuclear HIF-1α is directly phosphorylated by p42/p44 MAPKs, an action that enhances HIF-1-dependent transcriptional activation of VEGF. In addition, MAPKs activated in response to various stressors (p38MAPK and JNK) contribute to the increased expression of this angiogenic growth and survival factor by stabilizing the VEGF mRNA (5). A direct role of PI3K in regulating VEGF expression has been reported (25). Thus our results with kinase inhibitors support that copper shares some of the pathways utilized by hypoxia to induce VEGF expression. We have hypothesized that oxidation events may be involved in mediating the effects of copper on VEGF expression. A parallel was drawn by a recent paper reporting that hypoxia-induced VEGF expression is likely to be dependent on NADP oxidase-generated oxidants (14).

Lysyl oxidase (protein-lysine 6-oxidase; EC 1.4.3.13) is a copper-containing enzyme that functions extracellularly and catalyses the oxidative deamination of peptidyl lysine. Lysyl oxidase initiates the cross-linking of the lysine-derived aldehyde and plays an essential role in maturation of collagen and elastin during wound healing (22). It is thus clear that copper regulates multiple events that are central to the process of healing. Given that topical application of copper is simple and that copper is effectively absorbed by the human skin (4), copper-based approaches to promote dermal wound healing warrant further investigation in a clinical setting.

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