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ABSTRACT

Clinical observation suggests that oral mucosal wounds heal faster than skin; however, little is known about the site-specific differences. Since fetal skin wounds heal rapidly, but are less vascular than adult wounds, we hypothesized that less robust wound angiogenesis might be observed in healing oral mucosa. This study investigated angiogenesis in equivalent-size oral and skin murine wounds. Change in wound bed vascularity was significantly lower in oral wounds than in skin. Also, vascular endothelial growth factor (VEGF) levels were less in oral than cutaneous wounds. Because keratinocytes are a prominent source of VEGF in wounds, we compared VEGF production by oral and epidermal keratinocytes *in vitro*. Significantly higher levels of VEGF protein and mRNA were observed in epidermal keratinocytes than in oral keratinocytes after 18 hrs of hypoxia. This study demonstrates distinct angiogenesis patterns in oral and skin wounds and intrinsic site-specific differences in VEGF production by keratinocytes.

KEY WORDS: wound healing, angiogenesis, VEGF, oral mucosa, skin.

INTRODUCTION

Oral mucosa heals faster than skin, yet few studies have investigated differences in repair mechanisms at oral mucosal and skin sites. When hypoxia occurs locally, such as when perfusion is disrupted by injury, cells respond by increasing their production of VEGF (Knighton *et al.*, 1981). VEGF is the single most significant mediator of wound angiogenesis, and its production stimulates capillary growth to provide adequate nutrients, oxygen, and inflammatory cells. During wound healing, capillary density reaches more than twice that of uninjured normal tissue (Swift *et al.*, 1999), causing tissue oxygen levels to rise. Once normoxia is attained, the production of VEGF diminishes, signaling an end to the pro-angiogenic period. As healing resolves, many capillaries regress, resulting in a residual vascularity that is similar to that of uninjured tissue.

Wound angiogenesis follows an orderly pattern, yet its regulation is incompletely understood. Moreover, the precise requirements for wound angiogenesis are not known, since recent studies suggest that wound re-epithelialization can proceed normally even when angiogenesis is partially inhibited (Bloch *et al.*, 2000). Additionally, data regarding the pattern of wound angiogenesis in different anatomic sites, such as oral and dermal locations, are scarce.

The present study examined whether there are site-specific differences in wound angiogenesis. Utilizing a murine model of equivalent excisional dermal and oral mucosal wounds, we determined the pattern of neovascularization and the production of two critical angiogenic factors, VEGF and fibroblast growth factor-2 (FGF-2). In addition, we examined VEGF mRNA and protein levels *in vitro* in human oral and epidermal keratinocytes, since these cells are a major source of VEGF in healing wounds.

MATERIALS & METHODS

Animals and Wound Models

All animal procedures were approved by the Loyola University Institutional Animal Care and Use Committee. Female 6- to 8-week-old Balb/c mice (Harlan, Inc., Indianapolis, IN, USA) were anesthetized with pentobarbital injection (50 mg/kg), the dorsal skin was shaved, and 2 excisional dermal and oral mucosal wounds were made with the use of a 1-mm punch-biopsy instrument (Acu-Punch, Acuderm Inc., Ft. Lauderdale, FL, USA). Skin wounds were inflicted on the opposite sides of the midline at the scapula level. Oral mucosal wounds were inflicted on the dorsal surface of the tongue lateral to the midline. At various intervals after injury, wounds were removed, embedded in freezing medium or snap-frozen, and stored at -80°C for analysis.

Analysis of Wound Vascularization

Ten-μm sections were stained with an endothelial cell-specific monoclonal antibody to CD31 (PharMingen, San Diego, CA, USA) and counterstained with

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Harris' hematoxylin (Sigma, St. Louis, MO, USA) as previously described (Swift *et al.*, 1999). Sections were imaged by Optronics Acquisition software (Optronics Engineering, Goleta, CA, USA) and analyzed by ScionImage software (Scion Corp., Frederick, MD, USA). Using a freehand drawing tool, we outlined the wound bed and measured the wound area. Only the CD31-positive area within the wound bed was identified with a colorization tool in the software; vessel lumens were not colorized. Most vessels were capillaries with lumens of negligible size. At each time point, the % vascularization [CD31-positive area/Total wound bed area) X 100] was determined for 2 wound sections from each of 5 mice. We determined the increase in vascularization in the wound bed over the normal tissue by dividing by baseline values.

Analysis of Growth Factors in Wounds

VEGF and FGF-2 levels in wounds were determined by murine VEGF and human FGF-2-specific ELISA kits (R&D Systems, Minneapolis, MN, USA). The human FGF-2-specific ELISA kit (R&D Systems) has been previously shown to react with murine FGF-2 (Swift *et al.*, 1999; Szpaderska *et al.*, 2003). Briefly, wounds were excised by means of a 3-mm Acu-Punch and homogenized in 1.0 mL of PBS containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Homogenates were centrifuged and filtered through a 1.2-μm-pore filter. The growth factor levels were normalized to protein concentration.

Keratinocyte Isolation and Cell Culture

Keratinocytes were cultured at 37°C and 5% CO_2 in a humid atmosphere. Human adult skin keratinocytes were purchased from Clonetics and grown in keratinocyte basal medium-2, KBM-2 (Cambrex, Walkersville, MD, USA). Human oral mucosal tissue was obtained from healthy donors (age 15-25 yrs), after consent under a protocol approved by the Loyola University (Chicago) Institutional Review Board. Primary keratinocytes were isolated by a standard protocol that allows for the establishment of keratinocytes free of other cell types (Oda and Watson, 1990). The tissues were washed with PBS containing 50 μg/mL gentamycin and 0.5 μg/mL amphotericin B, and incubated overnight at 4°C with dispase solution (calcium- and magnesium-free PBS containing 25.0 caseinolytic U/mL dispase and 5 μg/mL gentamycin). Separated epithelium was incubated for 15 min at 37°C in 0.05% trypsin and 0.53 mM EDTA (Invitrogen, Carlsbad, CA, USA) so that a single cell suspension would be prepared. Following incubation, trypsin was neutralized with PBS containing 10 mg/mL Soybean Trypsin Inhibitor (Invitrogen, Carlsbad, CA, USA). The cell pellet was collected by centrifugation for 3 min at 700 rpm and re-suspended in KBM-2. Primary keratinocytes were seeded at a density of 3 x 10^6 cells *per* 75-cm^2 flask. At 75% confluence, KBM-2 was aspirated, and 1 mL of trypsin/EDTA solution was added. Cells were incubated for 10 min at 37°C, trypsin inhibitor was added, and the cell suspension was centrifuged for 3 min at 700 rpm. The cells were re-suspended in KBM-2 and transferred into a 60-mm Petri dish at a density of 1 x 10^5 cells *per* dish.

Analysis of VEGF Protein and mRNA in Keratinocytes

Skin and oral mucosal keratinocytes were plated in 6-well plates at a density of 1 x 10^5 cells/well in KBM-2 and incubated overnight. The following day, the plates were incubated for 18 hrs in hypoxic conditions. Hypoxia was generated in a tightly sealed chamber by means of AnaeroGen and assessed by Anerobic Indicator (Oxoid Ltd., Hampshire, England). Typically, the oxygen level in the chamber was reduced to below 1% within 30 min. The simultaneously generated carbon dioxide level was between 9% and 13%. For VEGF protein analysis, medium was removed, centrifuged at 1000 *g* for 10 min at 4°C, and immediately frozen at -80°C. A Quantikine Human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN, USA) was used to detect VEGF in culture supernatants. The cells were washed with PBS, detached with trypsin/EDTA, centrifuged at 1000 *g* for 3 min at 4°C, and re-suspended in PBS. Cell viability, determined by trypan-blue staining, was over 90% in all conditions. No evidence of death could be detected in cultures exposed to hypoxia, and cells continued to proliferate at a normal rate if re-exposed to normal oxygen tension. The experiment was done in triplicate.

For mRNA analysis, keratinocytes were rinsed with PBS and underwent lysis in TRI REAGENT (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Total RNA was isolated and treated with DNase I (Invitrogen, Carlsbad, CA, USA); reverse transcription was performed with Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA) and an oligo-dT primer (Amersham Pharmacia, Piscataway, NJ, USA), as described previously (Szpaderska *et al.*, 2003). The optimal number of PCR cycles for each gene was determined. PCR products were separated by gel electrophoresis and scanned by densitometry. Densitometry values of the 493-bp PCR product corresponding to $VEGF_{165}$ were normalized to β-actin expression at each time point and compared with the highest value set to 100. Primer sequences were: VEGF (NM_003376) 5′TGGGTGCATT GGAGCCTTGCCTTGCTGCTC3′, 5′TCTGGTTCCCGAAA CCCTGAGGGAGGCTCC3′, β-actin (X03672): 5′GTGGGCCG CCCTAGGCACCA3′, and 5′CTCTTTGATGTCACGCACG ATTTC3′.

Statistical Analysis

The mean and standard error of the mean were calculated for each experimental group. Data described over time were analyzed by two-way ANOVA, followed by the Newman-Keuls *post hoc* test. Data described at single time points were analyzed by an unpaired *t* test.

RESULTS

Angiogenesis in Oral and Skin Wounds

To determine whether oral mucosal and skin wounds are characterized by different wound vascularity, we analyzed the vascular density (Fig. 1A). At both sites, the development of vessels was rapid within the first 7 days, reaching maximal vascularity at day 5 (Fig. 1B). However, the vessel density in oral wounds, as compared with vessel density of uninjured mucosa, increased 3.4-fold. In contrast, vessel density in skin wounds increased 11.5-fold above the uninjured skin vasculature ($p < 0.001$) (Fig. 1C).

VEGF and FGF-2 Production in Oral and Skin Wounds

Since VEGF and FGF-2 promote wound angiogenesis, we used ELISA to examine levels of both factors in wounds. VEGF levels in uninjured oral mucosa and skin were not significantly different. However, at all examined time points post-injury, the levels of VEGF in oral mucosa were less than those of the cutaneous wounds. VEGF levels peaked earlier in oral wounds, at 24 hrs, *vs.* 36 hrs in the skin. At both time points, VEGF levels in oral wounds were lower than those in skin wounds (106.6 ± 19.0 *vs.* 194.0 ± 23.6 pg/mg protein, $p < 0.001$, at 24 hrs and 82.7 ± 8.9 *vs.* 374.2 ± 27.0 pg/mg protein, $p < 0.001$, at

36 hrs) (Fig. 2A). No statistically significant differences between oral and skin wounds were found in FGF-2 levels (Fig. 2B).

VEGF mRNA and Protein Levels in Keratinocytes

Since keratinocytes are the major source of VEGF in the wound and produce pro-angiogenic factors in response to hypoxia, we investigated the VEGF mRNA and protein levels in isolated oral and dermal keratinocytes. Age-matched, pooled primary human oral and epidermal keratinocytes were subjected to hypoxic culture conditions. Oral keratinocytes grown under normoxic conditions expressed 1.6-fold more VEGF mRNA than epidermal keratinocytes, as assessed by RT-PCR (Figs. 3A, 3B). Within 18 hrs of growth under hypoxia, VEGF gene expression was significantly elevated in both types of keratinocytes. However, the VEGF mRNA level in oral keratinocytes increased only 2-fold *vs.* a 4-fold increase in skin keratinocytes ($p < 0.05$, hypoxia *vs.* normoxia). ELISA measurement of VEGF protein gave similar results. Under normoxia, oral and epidermal keratinocytes produced similar levels of VEGF (750 ± 110 pg/mL *vs.* 620 ± 60 pg/mL). Upon exposure to hypoxia, VEGF release by oral keratinocytes increased to 1725 ± 200 pg/mL *vs.* 2110 ± 250 pg/mL by epidermal keratinocytes ($p < 0.01$, hypoxia *vs.* normoxia) (Fig. 4). Overall, when subjected to hypoxia, epidermal keratinocytes produced higher levels of both VEGF protein and mRNA than did oral keratinocytes.

DISCUSSION

When tissue heals, newly sprouted capillaries form, and then eventually undergo regression and involution. This complex biological process is highly regulated and remains incompletely understood. While the role of angiogenesis in wound healing has been widely investigated, the importance of the process in tissue repair is controversial. Studies with several anti-angiogenic agents resulted in decreased vascularity in skin wounds, yet did not alter the quality of healing (Roman *et al.*, 2002). Wound

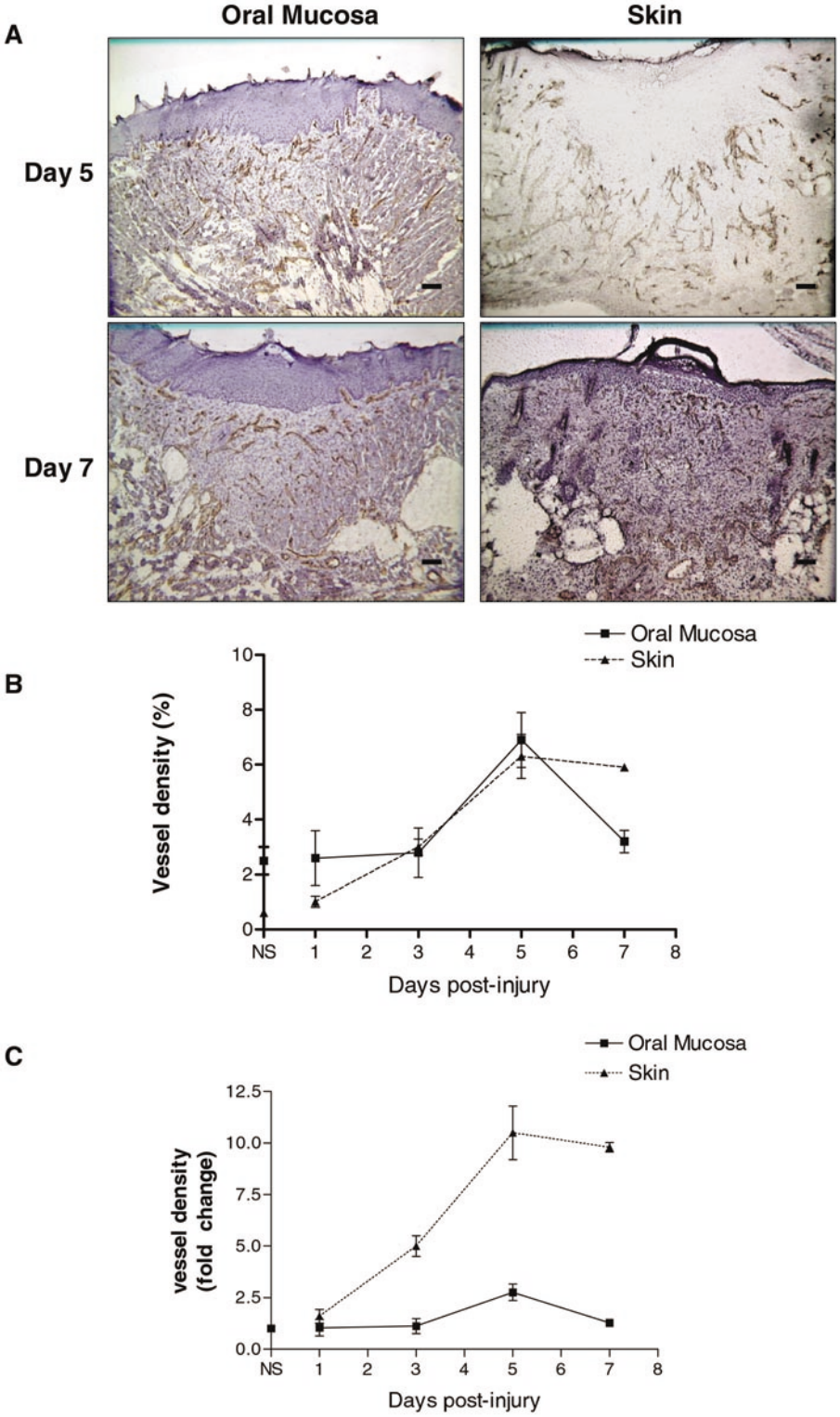

Figure 1. Time course of angiogenesis in oral mucosal and skin wounds. The vascular density of the wounds on days 1, 3, 5, and 7 after injury was determined by image analysis of CD31-stained tissues. Data are expressed as mean ± SE (n = 5). Scale bars (lower right corner of each panel): 200 μm. **(A)** Immunohistochemical localization of vessels in the wound bed was performed with anti-CD31 antibody. The photographs depict immunostained histologic sections of wounds from days 5 and 7 after wounding occurred. Original magnification x50. **(B)** Vessel density in normal skin and wound bed. The % vascularization [(CD31-positive area/Total wound bed area) x 100] was determined for 2 wound sections from each of 5 mice. **(C)** Increase in vessel density in wound bed over the baseline (normal tissue vasculature). We calculated the change in vascularity by dividing the vasculature value of each day by baseline value. Data are expressed as mean ± SE (n = 5). $p < 0.001$, mucosa *vs.* skin. NS, normal skin.

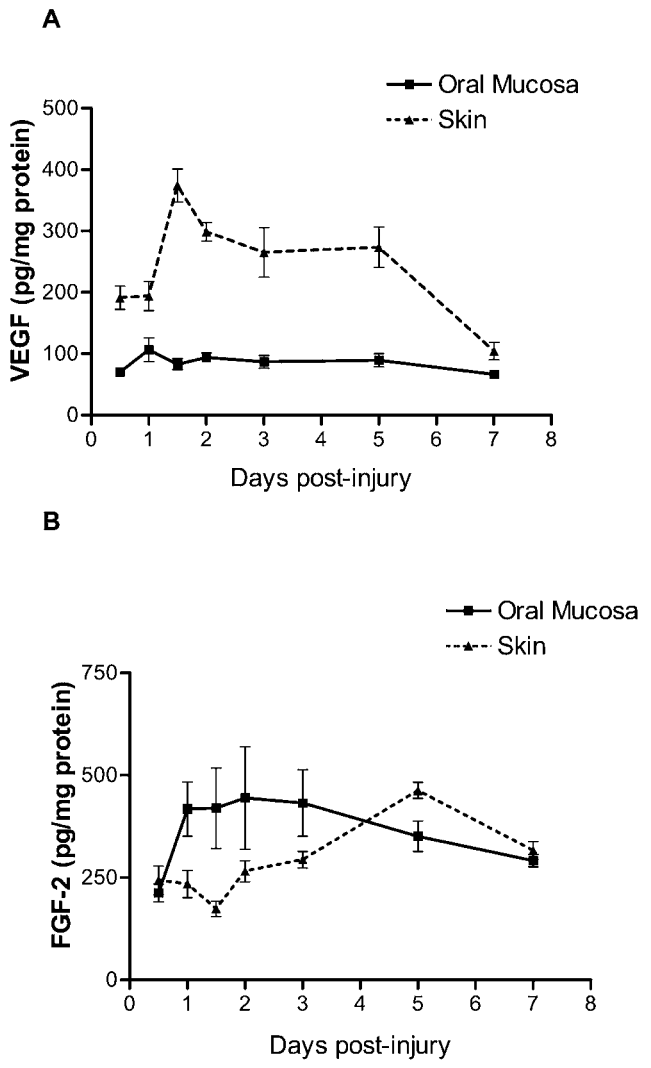

Figure 2. Levels of pro-angiogenic factors in oral mucosal and skin wounds. Wound tissue was collected and homogenized, and the supernatant was analyzed by ELISA. **(A)** VEGF (n = 4). $p < 0.001$, mucosa *vs.* skin. **(B)** FGF-2 (n = 4).

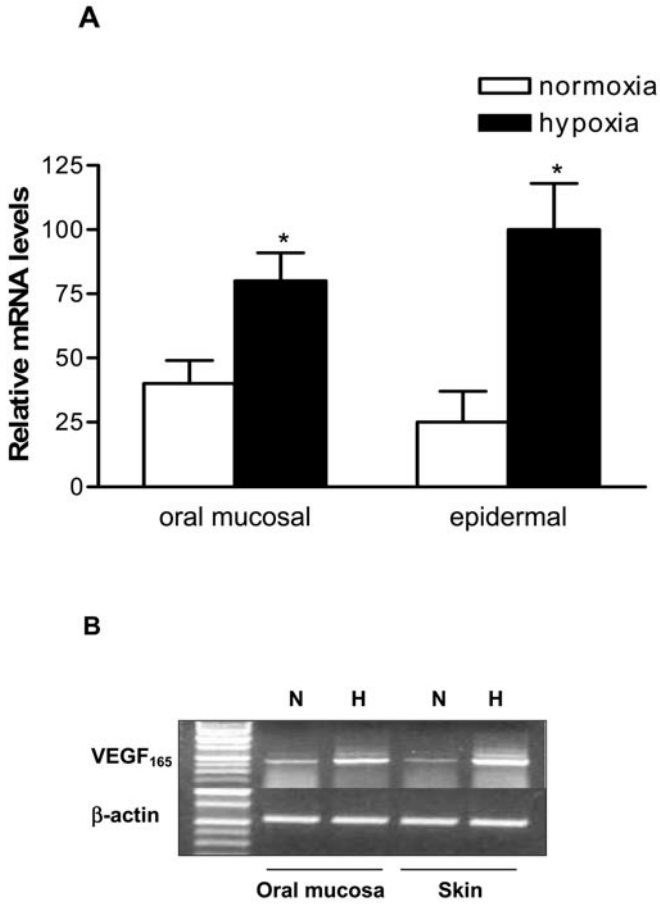

Figure 3. VEGF mRNA expression in oral and epidermal keratinocytes. **(A)** To determine relative changes in mRNA levels, we compared the densitometry values for 493-bp PCR product ($VEGF_{165}$) with β-actin expression and normalized them by setting the highest value to 100. The results are depicted graphically as the mean ± SE (n = 3). *$p < 0.05$ hypoxia *vs.* normoxia. **(B)** A representative gel of RT-PCR performed on RNA isolated from keratinocytes grown under normoxic and hypoxic conditions. N = normoxia, H = hypoxia.

healing in rabbits administered the angiogenesis inhibitor TNP-470 was not impaired (Tanaka *et al.*, 1996), and treatment with endostatin was described to have no effect on murine incisional skin wound healing (Berger *et al.*, 2000). In humans, endostatin treatment has been shown to have no effect on skin-punch biopsy healing (Mundhenke *et al.*, 2001). Contrary to these studies, a recent study in mice reported that treatment with angiostatin caused both a reduction of granulation tissue vascularity and an impaired healing response (te Velde *et al.*, 2003). Similarly, suppression of angiogenesis by orally administered inhibitors, TNP-470 and resveratrol, resulted in delayed skin wound healing in mice (Klein *et al.*, 1999; Brakenhielm *et al.*, 2001).

The requirement for angiogenesis in wounds remains controversial. However, one recent study suggests that robust angiogenesis supports scar formation. In this study, endostatin, administered subcutaneously to mice, led to impaired vessel integrity and reduced granulation tissue in excisional wounds. Scarring was slightly reduced, and the deposited collagen appeared less dense, demonstrating that endostatin treatment could improve the quality of the healed wound (Bloch *et al.*, 2000).

Our findings suggest that wounds with superior healing, such as oral mucosal injuries, may exhibit less robust neovascularization. Oral wounds heal quickly, and generally exhibit less scar formation than dermal wounds. Analysis of our data supports the hypothesis that robust angiogenesis seen in dermal wounds supports scar formation. The more modest angiogenesis observed in oral mucosal wounds may keep the fibrotic response in oral tissues in check by reducing available nutrient support.

The mechanism by which angiogenesis is regulated in oral tissues remains to be elucidated. However, our studies suggest that decreased production of VEGF may be a factor. Among the 30 or more known pro-angiogenic factors, VEGF has been shown to be the dominant mediator of wound angiogenesis (Nissen *et al.*, 1998). In normal skin, VEGF is expressed at low levels, whereas its expression is highly up-regulated in keratinocytes during healing (Berse *et al.*, 1992; Brown *et al.*,

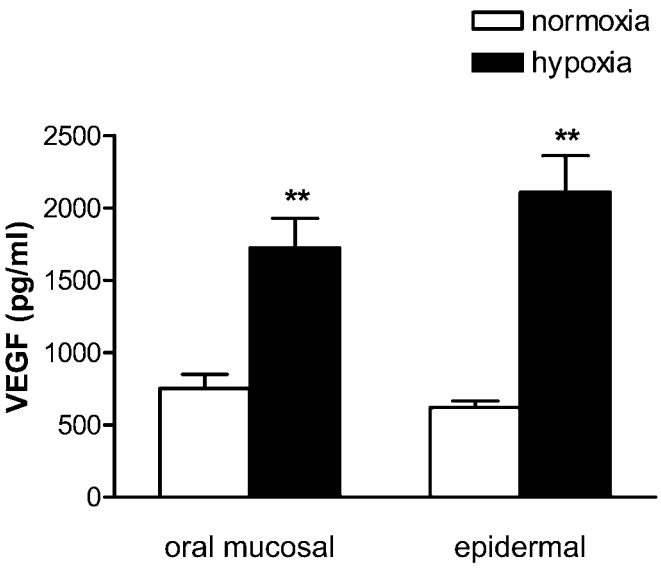

Figure 4. VEGF production by oral and epidermal keratinocytes. 1 x 10^5 oral and skin keratinocytes were incubated under hypoxia for 18 hrs. Culture supernatants were collected and analyzed (by ELISA) for VEGF release. As a control, VEGF protein concentrations were determined in media of cells grown under normoxia. The bars represent the mean ± SE (n = 3). **p < 0.01 hypoxia *vs.* normoxia.

1992; Kishimoto *et al.*, 2000). Our findings demonstrate that VEGF production in oral wounds lags behind the levels seen in skin. It has been suggested that the presence of VEGF in salivary glands and saliva may facilitate the high healing capacity shown by oral tissues (Pammer *et al.*, 1998; Taichman *et al.*, 1998). Despite the presence of VEGF in saliva, our studies indicate that the levels of VEGF within the wound are lower in oral than in cutaneous injuries at all time points. Similarly, our previous studies demonstrated that expression of TGF-β, another pro-angiogenic growth factor, is lower in oral wounds than in skin wounds (Szpaderska *et al.*, 2003). However, the site-related decrease in growth factors does not seem to be global, since no differences in FGF-2 levels were seen in oral and skin wounds. These findings suggest that oral mucosal wounds are characterized by a lower expression of some, but not all, pro-angiogenic factors than are skin wounds. Both VEGF and FGF-2 levels were very similar in oral mucosal and skin wounds 7 days after injury. However, vessel density was lower in oral wounds as compared with skin at this time point. This difference in angiogenesis may be due to the presence of other pro-angiogenic factors in the skin that we did not investigate. Since wound angiogenesis is dictated by the equilibrium between pro- and anti-angiogenic factors, the oral wounds may also have higher levels of inhibitors of capillary growth.

In the skin, migrating keratinocytes at the wound edge express high levels of VEGF, suggesting that keratinocyte-derived VEGF stimulates angiogenesis during wound healing (Brown *et al.*, 1992; Ballaun *et al.*, 1995; Detmar *et al.*, 1995; Frank *et al.*, 1995; Viac *et al.*, 1997). Results from the present study suggest that VEGF gene expression and protein production differ in oral and epidermal keratinocytes. Following a hypoxic stimulus, VEGF mRNA and protein levels increased about 2-fold, while epidermal keratinocytes exhibited a 3- to 4-fold increase. These intrinsic differences may represent at least part of the mechanism of reduced wound angiogenesis at oral sites.

Taken together, our studies and those of others suggest that, in normal wound closure, robust angiogenesis may be in excess of physiologic needs, and may in fact support scar formation. This hypothesis does not deny that a certain level of angiogenesis is likely required for optimal healing. Additional studies are necessary for full understanding of the significance and mechanism behind the site-specific differences in angiogenesis in oral mucosal and skin wounds.

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