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Loxosceles deserta SPIDER VENOM INDUCES THE EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN KERATINOCYTES

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Abstract—Evenomation by arachnids of the genus *Loxosceles* frequently results in disfiguring necrotic skin lesions. The cellular and molecular mechanisms which contribute to lesion development are incompletely defined but appear to involve participation of several pro-inflammatory mediators. We have recently observed that *Loxosceles deserta* venom induces the production of chemokines in human umbilical vein endothelial cells (HUVECs) and human pulmonary epithelial cells. In the present study we observed that *Loxosceles deserta* venom induces the expression of vascular endothelial growth factor (VEGF) in human keratinocytes but little in smooth muscle cells and none in pulmonary epithelial cells. A potent endothelial cell-specific mitogen, VEGF induces angiogenesis and vascular permeability in vivo. RNase protection assay data indicate that VEGF mRNA concentrations in keratinocytes are significantly increased at 2 h following venom exposure. These data suggest that keratinocyte-derived VEGF may contribute to the vasodilation, edema and erythema which occur following *Loxosceles* evenomation.

INTRODUCTION

The most important North American arachnid that causes tissue necrosis is *Loxosceles reclusa*, commonly known as the brown recluse spider (1). The clinical spectrum of brown recluse spider-induced wounds can range from a small lesion marked by local pain and erythema to a much more serious would marked by extensive, often refractory dermal necrosis. Occasionally, individuals bitten by *Loxosceles* spiders exhibit systemic manifestations including nausea, vomiting, fever, chills, arthralgia, hemolysis, renal failure, and even death (2).

Although experimental dermonecrotic lesions with *Loxosceles* venom are readily produced in guinea pigs and rabbits, the mechanism of venom action remains incompletely defined. Animal studies and in vitro analyses of venom-

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induced cell activation suggest that a variety of soluble proinflammatory mediators participate in lesion development. We recently observed that *Loxosceles deserta* venom is a potent inducer of chemokine production by both endothelial and epithelial cells (3). Furthermore, *Loxosceles deserta* venom-induced chemokine expression in HUVECs is mediated by members of the Rel/NF-κB family of transcription factors (4). VEGF (vascular endothelial growth factor), also designated VPF (vascular permeability factor) is a potent endothelial cell-specific mitogen which induces angiogenesis and vascular permeability in vivo. VEGF is expressed in normal epidermis. Overexpression of VEGF by epidermal cells has been reported in certain non-neoplastic processes characterized by increased microvascular permeability and angiogenesis, e.g. wound healing, psoriasis and diseases associated with blister formation (5). Accordingly, VEGF is believed to be a major skin-derived cytokine that regulates local dermal microvascular permeability and angiogenesis. In the studies presented herein we observed that *Loxosceles* venom specifically induces the production of VEGF in human keratinocytes but not in smooth muscle cells (SMCs) or pulmonary epithelial cells. These results suggest that *Loxosceles deserta* may be a potent inducer of VEGF expression in human skin which in turn may contribute to lesion formation.

METHODS

Loxosceles deserta venom was purchased from Spider Pharm, Feasterville, Pennsylvania. Recombinant human TNF-α and IL-1β were obtained from R&D Systems Minneapolis, Minnesota).

Endothelial Cell Culture. Human umbilical vein endothelial cells were isolated from umbilical cords by treatment with 0.1% collagenase in Dulbecco's phosphate buffered saline (Bio Whittaker, Walkersville, Maryland) as previously described (6). Cells were grown and maintained in M199 medium (Bio Whittaker) supplemented with 20% heat-inactivated fetal bovine serum, L-glutamine (1.3 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml), endothelial cell growth supplement (25 μg/ml) and bovine lung heparin (100 μg/ml). Cells were plated onto gelatin-coated 150 $\times$ 25 mm plates, allowed to grow to confluence at 37°C in 5% CO_2, and utilized between the first and third passage.

Human Aorta Vascular Smooth Muscle Cell Culture. Human aorta vascular smooth muscle cells were purchased from Clonetics (Walkersville, Maryland) and were grown and maintained in smooth muscle cell basal medium (SmBm) supplemented with human recombinant epidermal growth factor (hEGF, 10 μg/ml), human recombinant fibroblast growth factor (hFGF, 1 μg/ml), dexamethasone (0.39 mg/ml), gentamicin (50 mg/ml), amphotericin-B (50 μg/ml) and FBS (5%). The cells were used between the fifth and eighth passage.

Keratinocyte Culture. Pooled normal human epidermal keratinocytes from neonatal skin were obtained from Clonetics. They were grown and maintained in keratinocyte basal medium (KBM) which was supplemented with rhEGF (0.1 μg/ml), insulin (5.0 mg/ml), hydrocortisone (0.5 mg/ml), gentamicin (50 mg/ml), amphotericin-B (50 μg/ml) and bovine pituitary extract (0.4%). The keratinocytes were used between their first and fifth passage.

Pulmonary Epithelial (A549) Cell Culture. Human pulmonary epithelial A549 cells were purchased from ATCC (Manassas, Virginia) and were grown and maintained in M199 medium (Bio

Whittaker) supplemented with PSF (penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml), 1 mM glutamine, 25 mM HEPES and 15% fetal bovine serum.

Whole Cell (WC) Enzyme Immunoassay for VEGF. Cells were cultured on 96-well flat bottom polystyrene plates (Costar) and were used for experiments after reaching confluence. VEGF capture plates were prepared by coating sterile 96-well flat bottom ELISA plates (Corning, Corning, New York) with anti-human VEGF antibody (R&D Systems, 100 μl/well, 0.8 μg/ml in PBS). The plates were incubated at 4°C overnight, washed 5 times with Wash Buffer (0.05% Tween-20 in PBS, pH 7.4) and blocked overnight at 4°C with blocking buffer (0.05% Tween-20, 1% BSA in PBS, pH 7.4) to reduce nonspecific binding. Capture plates were again washed 5 times with wash buffer and stored at 4°C until use. Following an 8 h exposure of cells to cytokines or *Loxosceles* venom, conditioned cell media were collected. 100 μl aliquots of the conditioned cell media and human recombinant VEGF standards (R&D systems) were loaded onto capture plates and incubated at 4°C overnight. Plates were washed 5 times with wash buffer. Biotinylated anti-human VEGF detection antibody (R&D Systems) was added to each well (0.1 μg/ml, 100 μl per well) and plates were incubated at room temperature for 2 h. After 3 washes, streptavidin horseradish peroxidase was added (Neutralite avidin, Southern Biotechnology Associates, Birmingham, Alabama; 100 μl/well, 1 : 4000 dilution) and incubated for 30 min at room temperature. Wells were washed 4 times to remove unbound conjugated peroxidase and then incubated with 100 μl/well of peroxidase substrate (ABTS®, Boehringer Mannheim Biochemica, Germany). After 0.5–4 h absorbance at 405 nm was measured using an automated microplate reader (EL_X808, Bio-Tek Instruments, Winooski, Vermont). VEGF concentrations were calculated by 4 parameter curve fitting or linear regression analysis of rhVEGF standard curves using KC3 software (Bio-Tek Inc.).

RNase Protection Assay. Total RNA was extracted from keratinocytes using Tri Reagent (Sigma) according to manufacturer's instructions. The RNase protection assays were performed using the RiboQuant Multi-probe kit from Pharmingen (San Diego, California) and carried out according to manufacturer's directions. Briefly, angiogenesis probes were radiolabeled with [γ =^{32}P] UTP (Amersham) and hybridized with the extracted total RNA at 56°C overnight. RNase digestion was carried out at 30°C for 45 min to degrade unhybridized single-stranded and excess probe and terminated by the addition of Proteinase K. After phenol/chloroform extraction and ammonium acetate/ethanol precipitation, the hybridized RNA probes were denatured at 90°C for 3 min and loaded onto a 4.75% denaturing polyacrylamide gel which was run in 0.5 X TBE at 50 watts constant power overnight. Gels were vacuum dried at 50°C for 90 min, placed on Kodak X-OMAT film in an autoradiography cassette with an intensifying screen and developed at −70°C.

Statistical Analysis. All values are expressed as the means ± standard error. Data were analyzed using Friedman two way analysis of variance (ANOVA) with Bonferroni comparison of means. Probability (P) values of <0.05 were considered significant.

RESULTS

IL-1β but not TNF-α or Loxosceles deserta *Venom Induces VEGF Expression in A549 Cells.* In the presence of medium alone, A549 cells produced a small quantity of VEGF over an 8 h period (Figure 1). Exposure to 0.2, 2.0 and 20.0 ng/ml of interleukin-1β (IL-1β), respectively, resulted in significant increases in VEGF secreted into the medium while cells that had been exposed to tumor necrosis factor-α (TNF-α, 0.2–2.0 ng/ml) did not produce significantly higher concentrations of VEGF than cells exposed to medium alone. A549 epi-

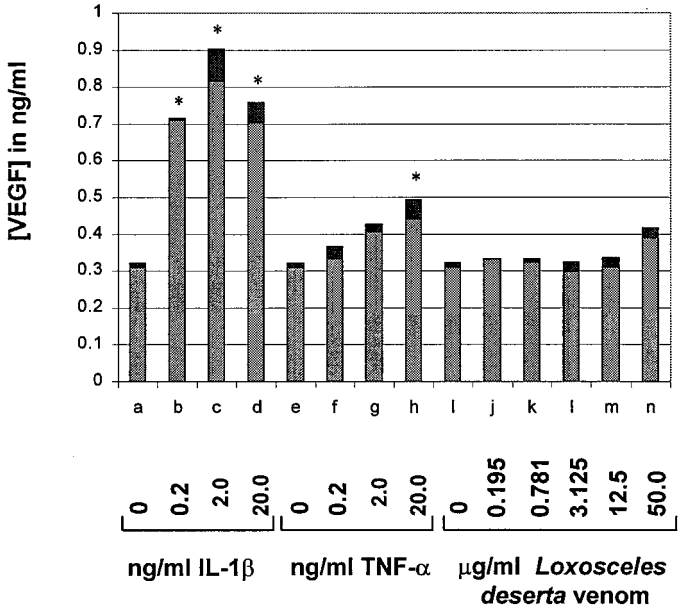

Fig. 1. VEGF secretion by A549 human pulmonary epithelial cells in response to IL-1β (lanes a-d), TNF-α (lanes e-h), and *Loxosceles deserta* venom (lanes i-n). ANOVA $P = 0.0016$ vs. controls. Asterisk* indicates significant difference from controls as determined by Bonferroni comparison of means.

thelial cells incubated in the presence of *Loxosceles deserta* venom (0.195–50 μg/ml) did not secrete significantly higher concentrations of VEGF than controls.

Loxosceles deserta *Venom Induces Low Levels of VEGF Expression in Human Aorta Vascular Smooth Muscle Cells.* Smooth muscle cells produce VEGF in response to a variety of cytokines and growth factors (7). Exposure of human aorta vascular smooth muscle cells to IL-1β resulted in marked dose-dependent VEGF secretion whereas TNF-α produced only moderate increases (Figure 2). Exposure of SMCs to *Loxosceles deserta* venom in doses ranging from 0.391 to 25 μg/ml resulted in modest increases in VEGF secretion at only 2 concentrations of venom (0.781 and 25 μg/ml). However, even at these concentrations, the quantity of VEGF secreted by venom-activated smooth muscle cells was much lower than the quantity induced by IL-1β.

Loxosceles deserta *Venom is a Potent Stimulus for VEGF Secretion in Human Keratinocytes.* Exposure of keratinocytes to the cytokines IL-1β or TNF-α at doses ranging from 0.2 to 20 ng/ml, respectively, failed to induce significant increases in VEGF concentrations (Figure 3). Incubation of keratinocytes

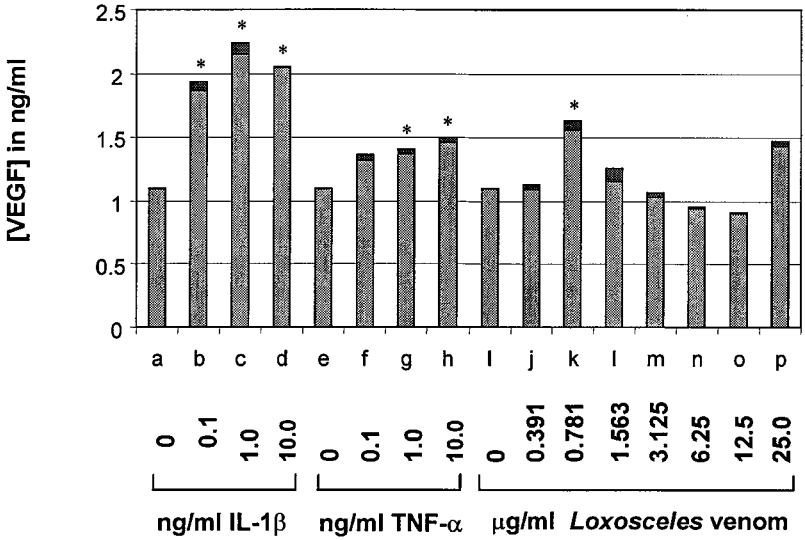

Fig. 2. Effects of IL-1β (lanes a-d), TNF-α (lanes e-h), and *Loxosceles deserta* venom (lanes i-p) on VEGF expression in human aorta vascular smooth muscle cells. ANOVA $P = 0.0004$ vs. controls. Asterisk* indicates significant difference from controls as determined by Bonferroni comparison of means.

with *Loxosceles deserta* venom at concentrations ranging from 0.195 to 50 μg/ml, resulted in marked, dose-dependent increases in VEGF concentrations. At a dose of 3.125 μg/ml, VEGF concentrations were increased by more than 200% above control values.

Exposure to Loxosceles deserta *Venom Results in Increased Concentrations of VEGF mRNA in Keratinocytes.* In order to determine whether increased synthesis of VEGF mRNA was induced in venom-activated keratinocytes, we utilized a multi-probe RNase protection assay. The assay included probes for VEGF-C, an angiogenic factor that is structurally and functionally related to VEGF (8). Figure 4 depicts the time course for the appearance of VEGF mRNA in keratinocytes incubated in presence of 3.125 μg/ml *Loxosceles* venom. Low concentrations of VEGF mRNA are present in unstimulated keratinocytes (lane 1). At 2 h following exposure to *Loxosceles deserta* venom (lane 2) VEGF transcripts were present in concentrations significantly increased over concentrations of VEGF mRNA observed in keratinocytes exposed to medium alone (lane 1). At 4 h, VEGF mRNA concentrations returned to background (lane 3) and remained at background levels even at 6 h following exposure to *Loxosceles* venom (lane 4). Consistent with the results from ELISA experiments (Figure 3), VEGF transcripts were not significantly increased in response to TNF-α (Fig-

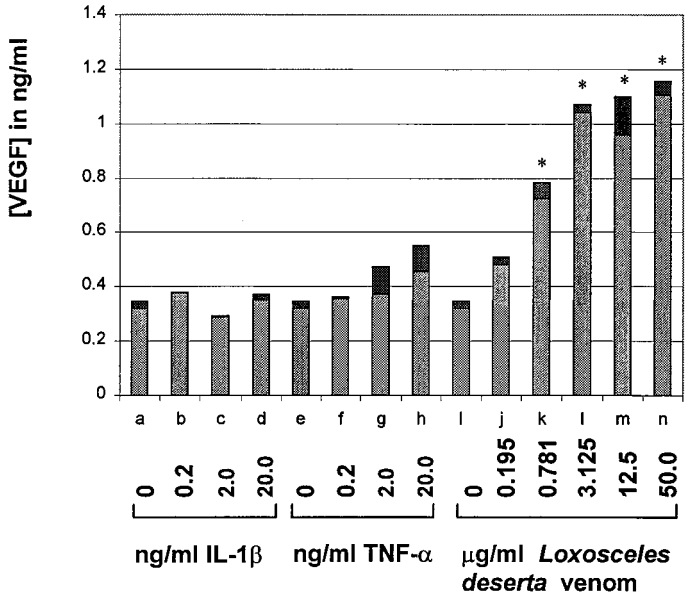

Fig. 3. VEGF secretion by human keratinocytes in response to increasing concentrations of IL-1β (lanes a-d), TNF-α (lanes e-h), and *Loxosceles deserta* venom (lanes i-n). ANOVA $P = 0.0041$ vs. controls. Asterisk* indicates significant difference from controls as determined by Bonferroni comparison of means.

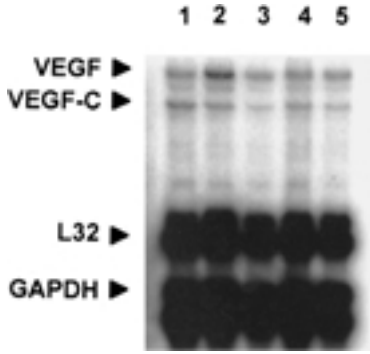

Fig. 4. Induction of VEGF mRNA in *Loxosceles deserta*-activated keratinocytes. RNase protection assays were utilized in order to detect specific transcripts for VEGF, VEGF-C and for the "housekeeping" genes L32 and GAPDH as controls. Keratinocytes were exposed to 1. Medium only (lane 1) 2. Venom for 2, 4, and 6 h, respectively (lanes 2–4) or 3. TNF-α (lane 5).

ure 4, lane 5). No significant changes in the concentrations of VEGF-C mRNAs were observed, suggesting that the increase in VEGF mRNA concentrations in keratinocytes is a specific response to venom exposure.

DISCUSSION

We previously observed that endothelial and pulmonary A549 epithelial cells exposed to *Loxosceles deserta* venom secrete a variety of chemokines including monocyte chemoattractant protein-1 (MCP-1), growth-related oncogene-alpha (GRO α) and interleukin-8 (IL-8) (3). Patel et al. reported that exposure of HUVECs to *Loxosceles* venom results in the induction of E-selectin and granulocyte-macrophage-colony-stimulating factor (GM-CSF) (9).

In vivo studies indicate that within 6 h following *Loxosceles* evenomation, both epidermal and dermal edema are observed. Histopathologic findings include the marked infiltration of venular walls by neutrophils, vasodilation, intravascular thrombosis, and massive hemorrhage into the adjacent dermis, subcutis, and sometimes into adjacent muscle. Bite sites are further characterized by fibrinoid necrosis and vacuolization of arteriole wall cells with resultant disruption of arteriolar integrity (10). VEGF, also known as VPF, is a potent endothelial cell mitogen which induces angiogenesis in vivo (11). On a molecular basis VEGF is approximately 50,000-fold more potent in inducing vascular permeability than histamine (12). The structural basis of the induction of vascular permeability by VEGF is not well understood but it has been speculated that upon binding of VEGF to its receptors, cytoskeletal changes occur which result in cell contraction and increased intercellular vascular permeability (11). VEGF has also been shown to induce fenestrations in endothelial cells in vivo and thus convert endothelium from a non-fenestrated into a fenestrated phenotype (11).

Some of the activities of VEGF can be considered to be pro-inflammatory (13). For example, enhanced vascular permeability is one of the earliest events in the inflammatory response. Moreover, VEGF is a chemoattractant for monocytes. Finally, VEGF has been shown to enhance the activity of TNF-α in the regulation of the expression of at least 2 endothelial cell proteins, tissue factor and Glut-1 glucose transporter (13). Several reports indicate that VEGF is expressed in human dermis where it may act as a regulator of vascular homeostasis (5). Increased expression of VEGF has been observed in the epidermis of healing skin wounds (12), in psoriatic skin lesions (14) and in bullous diseases associated with subepidermal blister formation (15).

Keratinocytes constitute approximately 95% of the cell mass of human epidermis and have increasingly come to be recognized as important regulators of inflammation (12, 16). Keratinocytes contain and express cytokines that promote

chemotaxis, activate macrophages and bone marrow elements, and induce lymphocyte homing (12). Recently Detmar et al. reported that transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), two factors that have been associated with the pathogenesis of wound healing and/or psoriasis, as well as phorbol myristate acetate (PMA), markedly stimulate VEGF mRNA expression and VEGF secretion in cultured keratinocytes (17). The VEGF secreted by keratinocytes was biologically active in that it is a potent stimulus of dermal endothelial cell proliferation.

Studies with genetically diabetic db/db mice that are characterized by a severe delay in skin repair have identified VEGF as an important cytokine participating in wound healing (18). During the healing process, continued vascular permeability results in extravasation of fibrinogen that serves as a provisional matrix and promotes angiogenesis and scar formation (12). However, the wound healing defect in db/db mice is associated with reduced levels of VEGF during skin repair suggesting that up-regulation of VEGF is essential for normal wound healing (18).

In the present study we observed that human keratinocytes but not human aorta smooth muscle cells or epithelial cells secrete high concentrations of VEGF in response to *Loxosceles deserta* venom. The concentration of VEGF produced in presence of venom greatly exceeds that produced by either TNF-α or IL-1β (0.2–20 ng/ml) stimulation of keratinocytes. In addition, exposure of keratinocytes to *Loxosceles* venom results in a significant increase in VEGF mRNA concentrations at 2 h following exposure to venom. These data suggest that following *Loxosceles* evenomation, VEGF expression may be rapidly upregulated in the dermis where it may act in concert with other pro-inflammatory mediators such as MCP-1, IL-8, E-selectin and GM-CSF. At least in part, VEGF may be responsible for the edema and erythema seen early after evenomation. Although not addressed in the present study, these observations may also suggest a role for VEGF in the wound healing process following *Loxosceles* evenomation.

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