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Loxosceles SPIDER VENOM INDUCES THE PRODUCTION OF α AND β CHEMOKINES: Implications for the Pathogenesis of Dermonecrotic Arachnidism[1]

HERNAN F. GOMEZ,[2] MARK J. MILLER,[3] ANJALI DESAI,[3] and JEFFREY S. WARREN[3]

[2]*Department of Surgery*
Section of Emergency Medicine
[3]*Department of Pathology*
University of Michigan Medical Center

Abstract—Bites from the brown recluse spider and other *Loxosceles* arachnids result in dermonecrotic skin lesions. Neutrophils (PMN) are essential to the development of *Loxosceles*-induced skin lesions, but paradoxically, in vitro PMN activation is inhibited by direct exposure to *Loxosceles* venom. Neutrophil activation occurs in response to a myriad of soluble mediators that include members of both the α and β chemokine families. Because arachnid envenomation results in the exposure of several different cell types to venom, we investigated venom-induced expression of α and β chemokines in both endothelial cells (human umbilical vein; HUVEC) and epithelial cells (A549 pneumocytes). Chemokine-specific capture enzyme immunoassays (EIA) were used to measure *Loxosceles deserta* venom-induced α chemokines: interleukin-8 (IL-8), growth-related oncogene-alpha (GRO-α), and β chemokines: monocyte chemoattractant protein-1 (MCP-1), and regulated on activation, normal T cell expressed and secreted (RANTES) in cell-free conditioned media from HUVEC and A549 cell monolayers. Exposure of HUVECs (8 h) to *Loxosceles* venom resulted in the production of IL-8 (5.2 ± 1.30 ng/ml), MCP-1 (1.44 ± 0.11 ng/ml) and GRO-α (1.97 ± 0.15 ng/ml) in a dose and time-dependent manner. Exposure of A549 cell monolayers to venom resulted in IL-8 (7.74 ± 0.30 ng/ml), and MCP-1 (2.61 ± 0.31 ng/ml), but neither GRO-α nor RANTES accumulated during an 8-hour incubation period. Chemokines accumulated in a venom dose and time-dependent manner. Neither cell type secreted RANTES in response to *Loxosceles* venom. These data indicate that *Loxosceles* spider venom is a potent inducer of α and β chemokines in both endothelial and epithelial cell types. Based on the established roles of IL-8, MCP-1, and GRO-α, in inflammation, these observations have relevance to the pathophysiology of *Loxosceles*-Induced dermonecrosis.

INTRODUCTION

Loxosceles arachnids are indigenous American spiders which possess a venom capable of causing intense dermal inflammation and disfiguring necrotic ulcers

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(1,2). In some cases the bite site may resemble pyoderma gangrenosum or other neutrophilic dermatoses (3). Among the 13 recognized species of *Loxosceles* that are found in the United States, *L. reclusa*, commonly called the brown recluse spider, is the major species responsible for envenomation. Most *Loxosceles* envenomations occur in the south central regions of North America (4). Although the characteristic skin lesion was first described by Caveness (5) in 1872, the mechanism of dermal inflammation and necrosis has not been fully elucidated. *Loxosceles* bites are characterized by PMN infiltration, platelet thrombi, and thrombosis (6). The key role of PMNs in the development of *Loxosceles* lesions was demonstrated by Smith et al., who found that depletion of circulating neutrophils in rabbits abrogated early leukocyte infiltration and hemorrhage (7). Neutrophils however, do not appear to be directly activated by venom, and paradoxically, appear to be inhibited by direct exposure to venom (8). The dermal inflammation that results from *Loxosceles* bites may persist for weeks to months, long after the venom would likely be present at the bite site (24).

Endothelial cell activation and neutrophil-endothelial adhesion are pivotal events in the acute inflammatory process. These processes are mediated by a diverse array of cell surface and soluble mediators including numerous cytokines and both endothelial and leukocyte adhesion molecules (9–11). Patel et al. reported that HUVECs exposed to *Loxosceles* venom release substantial amounts of IL-8 and granulocyte/macrophage-colony-stimulating factor (GM-CSF), and that they weakly express E-selectin (12). We recently observed that infusion of monoclonal antibody (MAb) to IL-8 attenuates the development of venom-induced ulcers in rabbits when compared to animals treated with either saline or isotype identical MAb controls (13). Human endothelial and epithelial cells can produce a wide variety of chemokines and cytokines (14–17). In view of the often intense acute, and chronic dermal inflammation observed at sites of *Loxosceles* envenomation, we investigated the spectrum and characteristics of chemokines produced by monolayer endothelial (HUVEC) and epithelial cells (A549) exposed to venom.

MATERIALS AND METHODS

Reagents. *Loxosceles deserta* spider venom was purchased from the Sigma Chemical Co. (St. Louis, Missouri). Human IL-8, GRO-α, MCP-1, and RANTES paired capture and detection antibodies were obtained from R&D Systems (Minneapolis, Minnesota D8050, DGROO, DCPOO, DRNOO, respectively). Unless otherwise indicated, all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri). Human tumor necrosis factor (TNF-α) was purchased from R&D Systems (Minneapolis, Minnesota 210TA). Lipopolysaccharide (LPS) was purchased from Sigma (L-3129 from *E. coli* 0127:B8). Enzyme immunoassays (EIA) assays were carried out in 96-well microtiter plates, read with a model Elx808 microplate reader (BIO-TEK Instruments,

Inc.; Winooski, Vermont), and analyzed using the KC3 software package (BIO-TEK Instruments, Inc.) for standard curve fitting and statistical analysis.

Human Umbilical Vein Endothelial Cells. HUVECs were isolated from umbilical veins by treatment with 0.1% collagenase in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Walkersville, Maryland), plated at 5×10^4 cells/well on gelatin-coated 96 well plates, and allowed to grow to confluence at 37°C in 5% CO_2 as previously described (18,19). Cells were grown in M199 medium (Whittaker Bioproducts) supplemented with 20% heat-inactivated fetal calf serum, L-glutamine (4mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 25 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, Massachusetts) and 15 U/ml bovine heparin. Cells were characterized by a cobblestone appearance and utilized between the first and third passages.

A549 Epithelial Cells. A549 cells are a transformed pulmonary epithelial cell line. A549 cells from ATCC (Rockville, Maryland CCL 185) were maintained in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Walkersville, Maryland) supplemented with 8% fetal calf serum in ventilated tissue flasks. In experiments outlined below, cells were detached from the plastic by incubation in 0.05% trypsin and 0.01% EDTA for 15 min and plated at 10^5 cells/cm^2, on the day before venom exposure. The cells were confluent at time of venom exposure.

Endothelial (HUVEC) and epithelial (A549) Cell Stimulation and Chemokine Measurements. Where indicated, confluent HUVEC or A549 monolayers were washed with serum free medium (SFM) and then exposed to venom at concentrations of either 2, 0.5, 0.125 or 0.031 μg/ml for time intervals of 0.5, 2 or 8 h. Venom-induced chemokine production was compared to production by either HUVECs or A549 cells exposed to the soluble mediators; tumor necrosis factor (TNF-α) (500 unit/ml), and lipopolysaccharide (LPS) (1 μg/ml). Negative controls included HUVECs or A549 cells exposed to either heat-inactivated venom (HI) (confirmed inactive by inability to induce skin lesions in rabbits) or SFM alone. After exposure to venom, aliquots of conditioned medium from cell cultures were assayed for the α chemokines, IL-8 and GROα, and the β chemokines, RANTES and MCP-1. Chemokine concentrations were determined by chemokine-specific capture EIAs (see below).

Enzyme Immunoassays for IL-8, GROα, MCP-1 and RANTES. The EIA method used for this investigation is a modification of the enzyme-linked immunosorbant assay (ELISA) procedure to detect soluble antigens (20). Standard 96-well, flat-bottomed microtiter plates (Corning Glass Works, Corning, New York) were coated with murine monoclonal IgG_1 antibodies (R&D Systems) specifically directed against each chemokine to be measured (IL-8, GROα, MCP-1, RANTES). After overnight binding of the capture antibody (4 μg/ml in PBS, pH 7.4), plates were incubated with blocking buffer (PBS pH 7.4, 1% BSA, .05% Tween 20) for 2 h and washed twice (0.05% Tween 20 in PBS). Conditioned medium and standards were added in triplicate for a 2h incubation period. Plates were twice washed with buffer followed by the addition of the respective biotinylated goat polyclonal detection IgG antibody (IL-8 = 20 ng/ml, GRO-α = 1 μg/ml, MCP-1 = 1μg/ml, RANTES = 1 μg/ml, R&D Systems). After plates were washed (X4) with buffer, streptavidin horseradish peroxidase 1:4000 dilution (Neutralite, catalogue number 7200–05, Southern Biotechnology Associates, Inc., Birmingham, Alabama) was added for 30 min. Plates were then washed (X6) with EIA wash buffer, and finally exposed to ABTS substrate (Boehringer Mannheim Biochemica, Germany, #1 112 422) for 35–40 min. Finally, absorbance was read at 405 nm on a model EL_x808, BIO-TEK, Inc. microplate reader. Chemokine concentrations were calculated by 4 parameter curve fitting or linear regression analysis of chemokine standard curves using KC3 software (BIOTEK, Inc.).

Statistical Analysis. All values are expressed as the mean ± standard error. Capture EIA measurements were conducted in triplicate and individual experiments repeated four times with similar results. Data from the EIA experiments were merged for analysis. Statistical significance was based on analysis of variance (ANOVA) with Bonferroni comparison of means. A P-value of <0.05 was considered significant (21).

RESULTS

Loxosceles Venom Induced Chemokine Production in HUVECs. Exposure of HUVEC monolayers to *Loxosceles* venom resulted in the progressive accumulation of IL-8 over an 8 h period (Figure 1). Analogous results were observed for MCP-1 and GRO-α (Figure 1). In contrast, no RANTES production was observed (Figure 1). Chemokines (IL-8, GRO-α, and MCP-1) secretion by HUVECs was venom dose-related (Figure 2). LPS, TNF-α, HI, but not SFM controls induced secretion of the chemokines tested (data not shown).

Loxosceles Venom-Induced Chemokine Production A549 Epithelial Cells. Exposure of A549 monolayers to *Loxosceles* venom resulted in the progressive accumulation of IL-8 over an 8 h period (Figure 3). Analogous results were observed for MCP-1. A modest but statistically insignificant accumulation of GRO-α was observed (Figure 3). In contrast, no RANTES production was observed (Figure 3). Chemokine (IL-8 and MCP-1) secretion by A549 monolayers was venom dose-related (Figure 4). LPS, TNF-α, HI, but not SFM controls induced secretion of the chemokines tested (data not shown).

DISCUSSION

Bites from the brown recluse spider and other *Loxosceles* arachnids result in necrotizing skin lesions that can be very disfiguring. Animal studies indicate

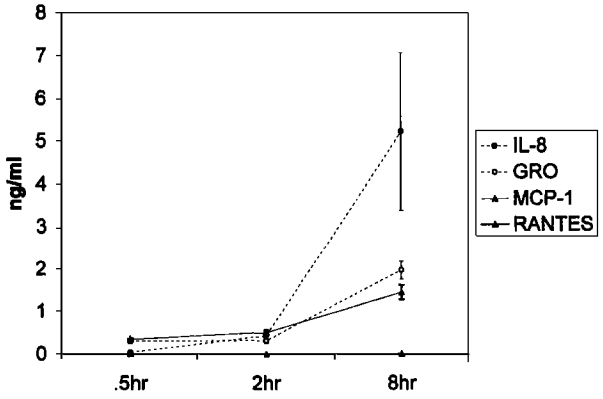

Fig. 1. IL-8, GRO-α, and MCP-1 accumulated in cell media over time in HUVEC conditioned media. Shown are HUVECs were exposed to *Loxosceles* venom 2.0 μg/ml. ANOVA $P < 0.04$ vs SFM and HI controls. Venom did not induce RANTES secretion by HUVEC monolayers.

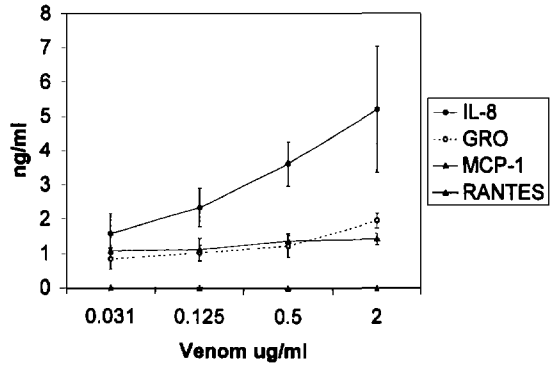

Fig. 2. IL-8, GRO-α, and MCP-1 secretion into HUVEC conditioned media was venom dose dependent. Accumulation values shown are following 8 h of venom exposure. ANOVA $P < 0.05$ vs SFM and HI controls.

that neutrophils are required for the development of *Loxosceles*-Induced dermonecrosis (7) and more recent in vitro studies indicate that *Loxosceles* venom can induce IL-8 and GM-CSF secretion by HUVECs (12). Although several studies have addressed the biochemical composition of *Loxosceles* venom, little is known about the pathophysiology of *Loxosceles* venom-induced dermal injury. We examined the quantitative and temporal characteristics of *Loxosceles* venom-induced chemokine production by endothelial and epithelial cells. Exposure of *Loxosceles deserta* venom to both HUVECs and A549 cells resulted in a dose-

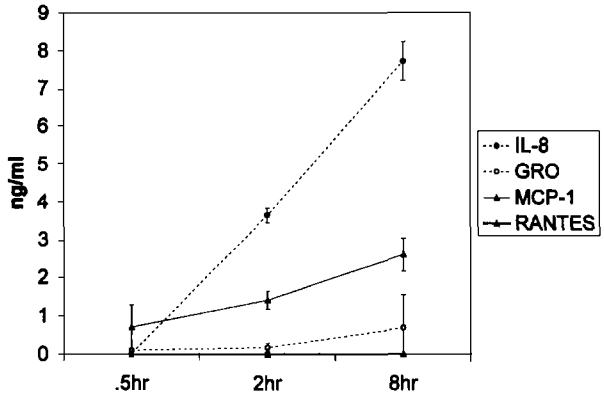

Fig. 3. IL-8, and MCP-1 accumulated in cell media over time in A459 conditioned media. Shown are A549 cells exposed to *Loxosceles* venom 2.0 μg/ml. ANOVA $P < 0.04$ vs SFM and HI controls. Venom did not induce RANTES or significant GRO-α secretion by A549 cell monolayers.

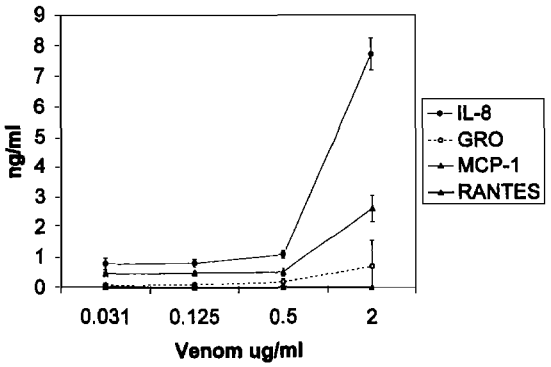

Fig. 4. IL-8 and MCP-1 secretion into A549 cell conditioned media was venom dose dependent. Accumulation values shown are following 8 h of venom exposure. ANOVA $P < 0.05$ vs SFM and HI controls.

dependent secretion of IL-8, GROα, and MCP-1. This indicates that both endothelial and epithelial cells are activated by *Loxosceles* venom and that a broad array of chemokines (both α and β) are induced by these two cell types. These data suggest that soluble α and β chemokines may participate in the development of *Loxosceles* venom-induced lesions. This suggestion is further supported by our recent observation that MAb-mediated neutralization of IL-8 reduces the dermonecrotic lesion size in rabbits that have received *Loxosceles* venom injections (13).

Previous studies have shown that neutrophils are essential to the development of *Loxosceles*-induced skin lesions (7), but the observation that human neutrophils are not directly activated by the venom in vitro suggests that endogenous mediators are likely involved in lesion development (8,22). The first ultrastructural change observed after envenomation is vascular endothelial damage which is followed by adhesion of neutrophils to the capillary wall (23). These observations suggest that activation of the vascular endothelium, with subsequent activation and recruitment of neutrophils is pivotal to the development of *Loxosceles*-induced skin lesions. In a series of in vitro studies using primary human endothelial cells, Patel et al. reported that *Loxosceles* venom can induce HUVEC synthesis and secretion of significant amounts of GM-CSF and IL-8, followed by adhesion and degranulation of neutrophils at endothelial intercellular junctions (12). We recently reported that IL-8 mediates the development of venom-induced skin lesions based on the observation that parentally administered monoclonal (MAb) to IL-8 attenuates the size of *Loxosceles* venom induced ulcers in rabbit skin when compared to saline and identical isotype MAb controls (13). Although we observed reductions in size of venom-induced ulcers in IL-8 MAb treated rabbits, ulcers nonetheless did occur, raising the possibil-

ity that other chemokines may play a significant role in the development of lesions.

There are limitations of this study. First, this is an in vitro study examining an extremely complex in vivo inflammatory pathologic process. As such, cells may respond differently to venom in a monolayer culture plate, than they might in an intact biological system. Although HUVECs and A549 cells are well-characterized examples of endothelial and epithelial cells respectively, it is possible that the various dermal cell types might respond differently to venom stimulation than did the cell types we studied.

The skin is an organ which contains a large variety of cell types. There is recent evidence suggesting that keratinocytes (15) and other types of epithelial cells are capable of synthesizing and releasing cytokines in response to a variety of stimuli (22–29). In view of previous in vitro work (12), in combination with the results of this investigation, the following model is proposed summarizing the cellular and molecular basis for tissue events postulated to occur following *Loxosceles* envenomation as follows: after envenomation dermal endothelial and epithelial cells produce and secrete the chemokines IL-8, GRO-α, and MCP-1. Endothelial cells secrete the cytokine GM-CSF and express the adhesion molecule E-selectin on the cell surface (12). Neutrophils migrate to the bite site and adhere to intercellular junctions via the E-selectin tethering protein (12). Neutrophils are subsequently activated and degranulate, thus resulting in tissue necrosis.

In our investigation we found significant synthesis and release of α and β chemokines by two relevant cell types commonly found in skin. We provide evidence that the severe dermal inflammation following *Loxosceles* spider bites is a complex form of inflammation, which may involve multiple cell types as well as multiple chemokines capable of contributing to this unique inflammatory process. Based on the roles of IL-8, MCP-1, and GRO-α, in inflammation, these observations have direct relevance to the pathophysiology of *Loxosceles*-induced dermonecrosis.

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