Published in final edited form as:

J Med Entomol. 2006 September ; 43(5): 910–915.

***Sarcoptes scabiei* (Acari: Sarcoptidae) Mite Extract Modulates Expression of Cytokines and Adhesion Molecules by Human Dermal Microvascular Endothelial Cells.**

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Abstract

The inflammatory and immune responses seen with the worldwide disease scabies (caused by the mite *Sarcoptes scabiei*) are complex. Clinical symptoms are delayed for weeks in patients when they are infested with scabies for the first time. This study was undertaken to elucidate the role of the human dermal microvascular endothelial cell (HMVEC-D) in modulating the inflammatory and immune responses in the skin to *S. scabiei*. Extracts of *S. scabiei* were incubated with HMVEC-D and the expression of adhesion molecules and chemokine receptors on the cells and the secretion of selected cytokines were determined by ELISA. *S. scabiei* extract was found to inhibit HMVEC-D expression of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) although not intercellular adhesion molecule-1 (ICAM-1). The secretion of interleukin-8 (IL-8) was also inhibited by *S. scabiei* extract. *S. scabiei* extract increased expression of the chemokine receptor CXCR-1, and both down-regulated and up-regulated expression of CXCR-2 depending on the concentration tested. These findings help explain the delayed inflammatory reaction to infestation with *S. scabiei*.

Keywords

Sarcoptes scabiei; scabies mite; vascular endothelium; adhesion molecule; chemokine

The disease “scabies” is a neglected but important worldwide health problem that is caused by the mite *Sarcoptes scabiei*, a mite which burrows in the stratum corneum of the skin. A primary infestation with scabies does not induce clinical manifestations in the skin for 4–8 weeks in humans (Orkin et al. 1977, Kemp et al. 2002, Elgart 2003). The mechanism responsible for the delayed primary response is not understood. However, substances from scabies mites have been shown to modulate the function of normal human cells including monocytes, dendritic cells, T-lymphocytes, epidermal keratinocytes and dermal fibroblasts (Arlian et al. 2003, 2004, 2006).

The endothelial cells of the microvasculature (post-capillary venules) of the skin play a key role in regulating migration of inflammatory and immune cells into the dermis. This is a precise, highly-regulated sequence of events in which leukocytes first adhere to the endothelial cells and then finally migrate into the tissues. The interaction of the leukocytes with the vascular endothelial cells lining the post-capillary venule is largely controlled by the regulated expression of the adhesion molecules intercellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2), vascular cell adhesion molecule-1 and -2 (VCAM-1, VCAM-2), E-selectin and P-

selectin and by chemokine receptors on the endothelial cells, secretion of chemokines and other cytokines by endothelial cells and other cells in close proximity (e.g., fibroblasts, keratinocytes) and counter-receptors on the leukocytes themselves in the blood. The appearance of inflammatory cells in the skin in the vicinity of a burrowing mite is associated with the manifestation of the clinical symptoms. The fact that clinical symptoms do not begin earlier in a primary infestation suggests that inhibition of the inflammatory and immune reactions may occur at least in part at the level of endothelial cells of the microvasculature of the skin. This may involve depressed secretion of cytokines and/or expression of surface adhesion molecules and chemokine receptors that regulate cell trafficking. The purpose of this research was to determine the expression profiles of the molecules of the human dermal endothelial microvasculature that regulate the sequence of events that result in leukocyte extravasation in response to products present in *Sarcoptes scabiei* extracts.

Materials and Methods

Sarcoptes scabiei mite extract

Extract was prepared from *S. scabiei* variety *canis* mites as described previously (Arlian et al. 2004). Briefly, mites of all life stages were collected, killed by freezing, and stored at -80°C . An aqueous whole body homogenate extract of *S. scabiei* was prepared in glass-distilled water at 1/20 (wt/vol). Mites were ground in a TenBroeck homogenizer on ice until smooth (10 strokes) and allowed to extract 24 h at 4°C . The soluble extract was collected by centrifugation, and the pellet was subjected to a second 24-h extraction. Pooled supernatants were sterile filtered ($0.22\text{ }\mu\text{m}$) into a sterile vial. Total protein concentration in the extract was measured by the Bradford protein assay (Bradford 1976).

Reagents

Biotinylated monoclonal antibodies directed against human ICAM-1 and VCAM-1 were purchased from eBioscience, Inc. (San Diego, CA). Biotinylated monoclonal antibodies directed against human E-selectin and ICAM-2 were purchased from R&D Systems, Inc. (Minneapolis, MN), as were nonbiotinylated monoclonal antibodies against the human chemokine receptors CXCR-1, CXCR-2 and CCR-5. Horseradish peroxidase-conjugated streptavidin (HRP-SA) and goat anti-mouse Ig were purchased from Fisher Scientific (Pittsburgh, PA). Preliminary experiments were performed to determine optimum antibody dilutions for each antibody (data not shown). Recombinant human tumor necrosis factor- α (TNF- α) was purchased from Endogen (Rockford, IL).

Endothelial cells

Cryopreserved adult dermal microvascular endothelial cells (HMVEC-D) and all media and growth factors were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, MD). Cells were cultured in Clonetics Endothelial Cell Basal Medium-2 supplemented with EGM-2-MV growth factors (EGM-2) at 37°C in 5–7% CO_2 . Cells were plated and grown to confluency in Costar 96-well culture plates (Corning, Inc., Corning, NY) for subsequent cell ELISA and cytokine secretion studies. All studies were performed on cells between passages 3 and 11.

Cell-ELISA procedure

The expression of cellular adhesion molecules and receptors was determined using a cellular-ELISA procedure modified from Yokote et al. (1993). HMVEC-D cells (3,000–6,000 cells per well) in 96 well plates were incubated in 200 μl of EGM-2 medium at 37°C in 5–7% CO_2 for 48–72 h until a confluent monolayer was reached. At the beginning of each experiment, 100 μl of medium was removed and discarded and replaced with 100 μl fresh medium containing

TNF- α at 4 ng/ml (final concentration) without or with *S. scabiei* extract at a specified concentration. Cells were reincubated for a specified time period (up to 48 h). At the conclusion of the incubation period the cell supernatants were removed, collected into microcentrifuge tubes, centrifuged at $14,000 \times g$, and the supernatants were collected and stored at -80°C . These cytokines were later assayed for cytokine production as described following.

To assay for the presence of cell surface markers, cells were washed with Dulbecco's phosphate buffered saline with 0.05% Tween 20 (PBST; Sigma Chemical Co., St. Louis, MO), and wells were blocked for 30 min at 37°C in 5–7% CO_2 with 300 μl endothelial cell basal medium containing 2% bovine serum albumin (EBM/BSA). EBM/BSA was removed, and 50 μl of the desired concentration of primary antibody (diluted in EBM/BSA) was added to each well. Wells were incubated for 30 min at 37°C in 5–7% CO_2 , then washed twice with PBST. Binding of biotinylated antibodies was detected by incubating the cells for 30 min at 37°C in 5–7% CO_2 in 50 μl of a 1:2,000 dilution (in EBM/BSA) of HRP-SA. Binding of nonbiotinylated antibodies was detected by incubating the cells for 30 min at 37°C in 5–7% CO_2 in 50 μl of a 1:1,000 dilution (in EBM/BSA) of HRP-labeled goat anti-mouse antibody. Wells were washed twice in PBST, and once in PBS. Fifty μl of TMB ELISA substrate (Sigma) was added to each well, and the plate was incubated for up to 15 min before color development was stopped with the addition of 50 μl of 1M sulfuric acid. Absorbance was measured at 450 nm using a BioTek EL800X microplate reader.

Cytokine assays

Culture supernatants collected at the conclusion of the stimulation of endothelial cells with TNF- α /*S. scabiei* were assayed for the presence of various cytokines. Endogen ELISA kits for detection of human monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), IL-1 α , IL-1 β , and IL-8 were purchased from Pierce Biotechnology Inc. (Rockford, IL). IL-6 ELISA kits were purchased from e-Bioscience (San Diego, CA). All assays were performed according to manufacturers' instructions. Absorbance was read using the BioTek EL800X microplate reader set to 450 nm.

Data analysis

Results of all assays were confirmed by one or more replicate experiments and representative data are shown. Statistical significance was determined using the two sample *t*-test with assumption of unequal variance and a *P*-value ≤ 0.05 was considered significant.

Results

Cellular adhesion molecules

HMVEC-D cells were assayed for expression of ICAM-1, ICAM-2, VCAM-1, P-selectin and E-selectin using the cell-ELISA procedure.

ICAM-1 was produced constitutively, but expression increased with the addition of TNF- α to the medium. Expression increased over time, with near-maximal expression present by 12 h of incubation with TNF- α (data not shown for 24 and 48 h incubations). Addition of *S. scabiei* extract to the medium with TNF- α did not have a significant effect on the level of expression of ICAM-1 (Fig. 1). ICAM-2 was not constitutively expressed or expressed when the cells were co-incubated with TNF- α and *S. scabiei* extract or *S. scabiei* extract alone (data not shown).

VCAM-1 expression by HMVEC-D cells was detected only in the presence of TNF- α , and reached near-maximum expression by 12 h. This TNF- α -stimulated expression of VCAM-1 was significantly inhibited in a dose-dependent fashion by *S. scabiei* extract (Fig. 1). Maximum

inhibition of 56% was achieved with 200 µg of *S. scabiei* protein/ml of cell culture medium after 9 h of incubation.

E-selectin expression was also dependent upon the presence of TNF- α in the medium, and was expressed earlier than ICAM-1 or VCAM-1 (Fig. 1). Maximum expression was present by 6 h, and decreased after that time. The TNF- α -induced expression of E-selectin was also significantly inhibited in a dose-dependant fashion by *S. scabiei* extract (Fig. 1). Maximum inhibition of 57% was seen at both 100 and 200 µg of *S. scabiei* protein/ml of culture medium after 9 h of incubation. Expression of P-selectin was not observed when cells were incubated without and with *S. scabiei* extract in the absence or presence of TNF- α for times ranging between 30 min and 48 h (data not shown).

Cytokines

Supernatants from cells incubated without and with *S. scabiei* extract in the absence and presence of TNF- α were assayed for IL-1 α , IL-1 β , MIP-1 α , IL-6, IL-8 and MCP-1. No IL-1 α , IL-1 β or MIP-1 α was detected in the supernatants tested.

IL-6 was produced in small quantities by HMVEC-D cells, and production was stimulated slightly by addition of TNF- α (data not shown). IL-6 production was not significantly affected at most *S. scabiei* extract concentrations tested, but there was an overall general increase in production with increasing quantities of scabies extract (Fig. 2).

MCP-1 and IL-8 were both constitutively produced in high quantities by HMVEC-D cells, with expression increased in the presence of TNF- α (data not shown). Co-incubation with TNF- α and *S. scabiei* extract did not have a significant effect on secretion of MCP-1 (Fig. 2). In contrast, TNF- α -induced secretion of IL-8 was significantly decreased in a dose-dependent fashion in the presence of *S. scabiei* extract (Fig. 2). IL-8 secretion was 85% inhibited when cells were treated for 6 h with 200 µg/ml *S. scabiei* extract; inhibition was 65% and 52% respectively after 9 and 12 h of incubation.

Chemokine receptors

HMVEC-D cells were assayed for the presence of CXCR-1, CXCR-2 and CCR-5. There was no detection of constitutive expression of these receptors nor did *S. scabiei* alone induce expression (data not shown). Expression of all three receptors was detected only after addition of TNF- α to the medium. Co-incubation with *S. scabiei* extract significantly increased expression of CXCR-1 at all concentrations of extract tested (maximum increase of 52% seen with 12 h incubation with 100 µg/ml of *S. scabiei* extract) (Fig. 3). TNF- α -induced expression of CXCR-2 was down-regulated by the lowest dose of *S. scabiei* while it was significantly increased at the highest *S. scabiei* concentration tested (Fig. 3). *S. scabiei* extract did not have a significant effect upon the expression of CCR-5 (Fig. 3).

Discussion

We found that extracts of *Sarcoptes scabiei* influenced expression of selected surface adhesion molecules and chemokine receptors and the secretion of chemokines in cultured human microvascular endothelial cells of dermal origin. Previous histological studies have shown that once the inflammatory reaction to *S. scabiei* has begun, neutrophils, Langerhans cells and lymphocytes make up a major portion of the cell infiltrate in the scabietic lesion, while few macrophages are seen (Arlian et al. 1994, 1996, 1997; Stemmer et al. 1996). This study was undertaken to provide insight into the reasons for the observed delay in time between infestation and the subsequent inflammatory response. In this study expression of the adhesion molecules E-selectin and VCAM-1 and secretion of the chemokine IL-8 were depressed by substances

in the scabies extract. Conversely, *S. scabiei* extract increased the expression of the IL-8 receptor CXCR-1 at all doses tested while it depressed the expression of CXCR-2 at a low dose and up-regulated it at a high dose. These results clearly indicate that secreted *S. scabiei* products or substances from *S. scabiei* bodies can modulate the inflammatory response in the skin.

Our results are consistent with those of a recent study in which tick salivary gland extracts were assessed for their ability to modulate the adhesion molecule expression of murine skin-derived endothelial cells (Maxwell et al. 2005). That study demonstrated that a salivary gland extract of *Dermacentor andersoni* contains components that down-regulate the TNF- α -induced expression of ICAM-1 *in vitro*. Likewise, a salivary gland extract of *Ixodes scapularis* reduced the TNF- α -stimulated expression of VCAM-1 and P-selectin.

E-selectin and P-selectin are expressed on the surface of activated endothelial cells and are known to mediate initial binding of neutrophils and other leukocytes to endothelial cells (Butcher 1991, Springer 1994). For our studies we used the proinflammatory cytokine TNF- α to activate skin endothelial cells because it is a general activator of endothelial cells that stimulates them to express surface adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 and multiple chemokine receptors, and to secrete various cytokines such as IL-8 (Woo et al. 2005). However, TNF- α does not activate endothelial cells to express P-selectin (Vestweber and Blanks 1999). Therefore we could not assess the effect of *S. scabiei* extract on the expression of P-selectin in activated dermal endothelium and its possible role in mediating the entry of leukocytes into a scabietic lesion. However, *S. scabiei* by itself did not induce expression of P-selectin. In this study we have also not evaluated how cytokines and other inflammatory mediators released from *S. scabiei*-stimulated cells in the dermis and epidermis (e.g. fibroblasts and keratinocytes) affect the expression pattern of adhesion molecules and/or chemokine receptors on endothelial cells or the secretion of cytokines by these cells.

E-selectin on the endothelial cell and IL-8 secreted by endothelial cells and cells in the vicinity of the mite (such as fibroblasts) both play key roles in the early phases of the migration of neutrophils from the blood vessel into the tissues. Expression of E-selectin and P-selectin on the endothelial cell contributes to the initial tethering and rolling leading to neutrophil extravasation (Springer 1994, 1995). IL-8 that binds to the endothelial cell surface activates neutrophils to increase the affinity of the neutrophil integrin receptors (e.g. LFA-1; lymphocyte function associated antigen-1 or MAC-1; integrin α M, β 2) for coupling with adhesion molecules on the endothelial cell surface which then allows the neutrophils to adhere firmly to the endothelial cell prior to transendothelial migration into the dermis. The down-regulated expression of E-selectin and IL-8 production seen here may contribute to the initial delay in the inflammatory response and clinical manifestations seen in a primary infestation with *S. scabiei*. The down-regulated expression of the VCAM-1 is probably less important in neutrophil migration than the down-regulated production of IL-8. VCAM-1 is generally thought to be more involved in the second step of leukocyte binding, resulting in tight bonds between lymphocytes, monocytes and eosinophils (rather than neutrophils) and the endothelial cell (Carlos and Harlan 1994, Springer 1994, 1995). Previous research has shown that fibroblasts up-regulate production of IL-8 in response to *S. scabiei* and this chemokine could be expected to be available to influence neutrophil migration (Arlian et al. 2003). While IL-8 binds to the receptors CXCR-1 and CXCR-2 on endothelial cells, the accompanying down-regulation of E-selectin by *S. scabiei* may prevent the necessary initial tethering and rolling, reducing the IL-8-induced effect of increased affinity between the neutrophil and endothelial cells that leads to transendothelial cell migration. *S. scabiei* extract by itself did not induce the expression of the IL-8 receptors CXCR-1 and CXCR-2 on HMVEC-D cells, however, in the presence of the inflammatory cytokine TNF- α these cells did express the receptors. CXCR-1 was up-regulated; CXCR-2 was both up and down-regulated depending on the concentration

of *S. scabiei* extract present. The significance of this is unclear in light of the accompanying down-regulation of IL-8 production by *S. scabiei*.

Neutrophil recruitment is facilitated by other factors such as C5a, leukotriene B4, and platelet activating factor (Vestweber and Blanks 1999). We have not examined if *S. scabiei* can modulate the expression or effects of these factors and thus further contribute to the inhibition of an early inflammatory response.

Circulating monocytes cross the endothelial wall of the venule and transform into macrophages in the dermis in the vicinity of a scabietic lesion. Circulating monocytes are tethered by selectins such as E-selectin and then selectively adhere more firmly to the endothelial cell via VCAM-1 in response to the chemokine MCP-1. We observed a *S. scabiei* dose-dependent inhibition of the expression of VCAM-1 but there was no *S. scabiei* effect on the secretion of MCP-1. Decreased expression of VCAM-1 coupled with no change in the level of expression of this monocyte-attracting chemokine may help to explain the paucity of macrophages found at the site of the scabies infestation once an inflammatory response has begun.

As the infestation proceeds, an inflammatory/immune response does eventually begin. Infestation typically begins with only one or a few mites. As the infestation progresses, the number of mites increases in the skin with accompanying increased production of antigenic material such as saliva, feces, and other secretions. In addition, mites in the epidermis begin to die and disintegrate in greater numbers, and these mite bodies are the source of hidden antigens. There is little of this hidden antigen early in an infestation when there are few mites in the skin. It may be that this increasing antigen load is responsible for shifting the balance toward an inflammatory/immune response after the initial delay. For example, previous studies have shown that the humoral immune response is triggered at the point when large numbers of mites have died in the skin (Arlian and Morgan 2000). Thus, it may be that the mite-controlled down-regulated response in the skin, as demonstrated in this study, is the adaptation that allows the parasite to initially become established on the host.

Acknowledgements

This study was funded by grant AI-017252 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases and by Wright State University institutional funds.

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Figure 1.

Effect of *S. scabiei* extract on the TNF- α -induced expression of cell adhesion molecules by human dermal microvascular endothelial cells. Cells were incubated with TNF- α (4 ng/ml) and varying doses of *S. scabiei* extract protein for 3, 6, 9, or 12 h as shown. Data are means + SEM for triplicate wells from one representative experiment.

Figure 2.

Effect of *S. scabiei* extract on the TNF- α -induced secretion of chemokines by human dermal microvascular endothelial cells. Cells were incubated with TNF- α (4 ng/ml) and varying doses of *S. scabiei* extract protein for 6, 9, or 12 h as shown. Data are means + SEM for duplicate wells from one representative experiment.

Figure 3.

Effect of *S. scabiei* extract on the TNF- α -induced expression of chemokine receptors by human dermal microvascular endothelial cells. Cells were incubated with TNF- α (4 ng/ml) and varying doses of *S. scabiei* extract protein for 12 or 24 h as shown. Data are means + SEM for triplicate wells from one representative experiment.