MODULATION OF CYTOKINE EXPRESSION IN HUMAN KERATINOCYTES AND FIBROBLASTS BY EXTRACTS OF SCABIES MITES

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Abstract. Sarcoptes scabiei lives in the stratum corneum of its mammalian host. Keratinocytes and fibroblasts are among the first cells to encounter the burrowing mite and its products. The aim of this study was to determine if molecules in an extract of S. scabiei modulate the expression of cytokines by keratinocytes and fibroblasts. Human keratinocytes and fibroblasts were exposed to an extract of S. scabiei var. canis in the absence or presence of Escherichia coli lipopolysaccharide. Cytokine expression was measured by an enzyme-linked immunosorbent assay. Components in the S. scabiei extract induced marked increases in secretion of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) and slight increases in production of granulocyte–colony-stimulating factor (G-CSF) by keratinocytes. The scabies extract down-regulated keratinocyte secretion of IL-1 receptor antagonist, but did not influence the production of IL-1α or IL-1β. In comparison, components in the scabies extract induced marked increases in the elaboration of IL-6, IL-8, G-CSF, and VEGF by fibroblasts. Neither cell type produced eotaxin, stem cell factor, or tumor necrosis factor-α under any of the conditions tested. This study demonstrates that components in an extract of the mite S. scabiei are able to influence cytokine expression by human keratinocytes and fibroblasts.

INTRODUCTION

The parasitic mite, Sarcoptes scabiei, resides in the stratum corneum of the epidermal skin of humans and many other mammals. Human patients infested with the mite develop and exhibit cutaneous inflammation and pruritus associated with the scabietic lesions. These clinical features develop slowly over time and as a result patients may not present symptoms until 6–8 weeks after they have contacted the parasite. Humans and other mammals with scabies exhibit strong dermal cellular infiltrates in the scabietic skin lesions and at some point in time have elevated antibody titers specific for antigens from the parasite. Some humans and dogs present with an allergic response and have circulating IgE to scabies antigens that frequently is cross-reactive with the phylogenetically related Dermatophagoides house dust mites. These inflammatory and immune reactions are likely driven by proinflammatory mediators and cytokines that are released by effector cells in the skin in response to products from the mite or physical stimulation of the burrowing mite.

Cells in the epidermis and dermis in the vicinity of the mite are the first to encounter products from the mite. Therefore, it is probable that keratinocytes, dendritic cells including Langerhans’ cells (LCs), and fibroblasts of the skin may initiate or play a key role in the induction of the inflammatory and immune reaction. In vitro studies show that keratinocytes in the epidermis of cultured human skin equivalents (HSE) release interleukin-1α (IL-1α) and IL-1β in response to burrowing mites. Addition of 10 ng of IL-6/mL or 3 and 30 ng of hepatocyte growth factor/mL in the culture medium of these parasitized skin equivalents results in up-regulated production of IL-1β. However, co-stimulation of the parasitized HSE with higher or lower concentrations of either of the two cytokines down-regulates the response to S. scabiei. In vivo studies show that there is a strong migration of LCs into the epidermis near scabietic lesions. Likewise, there is a strong migration of neutrophils, plasma cells, and T lymphocytes into the dermis. These studies clearly show that products from the mite modulate some aspects of inflammatory and immune cell responses. The events that occur in the epidermis and dermis of the skin that regulate the inflammatory/immune response are not well understood. These initial events are a key aspect of the immune defense against this parasite and how the parasite is able to withstand or elude the host response. The purpose of this study was to investigate if molecules in a scabies mite extract modulate the expression of cytokines from human keratinocytes and fibroblasts.

MATERIALS AND METHODS

Sarcoptes scabiei mite extract. Sarcoptes scabiei (SS) mite extract was prepared from S. scabiei var. canis (originally obtained from dogs) maintained on New Zealand White rabbits. An aqueous whole body homogenate extract of S. scabiei was prepared in glass-distilled water at a dilution of 1:20 (w/v). Total protein concentration in the extract was measured by the Bradford protein assay.

Human cells. Pre-plated (48 wells), normal human epidermal keratinocytes (NHEKs) and normal human dermal fibroblasts (NHDFs) from adult donors were obtained from Clonetics/BioWhittaker (Walkersville, MD). The normal human cell lines were cultured in their appropriate medium according to the supplier’s recommendations. The NHEK cells were cultured in keratinocyte growth medium 2 without Ca++. NHDF cells were cultured in fibroblast growth medium 2. Upon arrival, cells were allowed to equilibrate at 37°C in an atmosphere of 6% CO2 for 3–4 hours. After the cells had equilibrated, the shipping medium was removed and 1 mL of fresh medium was placed in all the wells. The cells then remained in the 37°C/6% CO2 incubator until they were ~70% confluent.

A series of preliminary experiments were performed to determine optimum concentrations of scabies extract and Escherichia coli lipopolysaccharide (LPS) for stimulating the cells. Time courses were also performed to determine the appropriate time interval for analyzing culture medium for cytokines during cell stimulation.

After cells became ~70% confluent, media was removed and NHEK and NHDF cells were challenged with fresh medium containing 50 μg of SS extract, 50 ng of E. coli LPS, or 50 ng of LPS plus 50 μg of SS extract. The SS extract was diluted in phosphate-buffered saline (PBS) to a concentration of 1 μg/μL and LPS was prepared in PBS to a concentration of 1 ng/μL so that each well contained 900 μL of medium plus 100 μL of additive(s) in PBS. As a control, cells of each
human cell line were given their appropriate culture medium (900 μL medium plus 100 μL of PBS). The cell culture supernatants were removed 24 hours later and assayed for cytokine expression.

**Determination of cytokine secretion.** Quantikine enzyme-linked immunosorbent assay kits for human IL-1α, IL-1β, IL-6, IL-8, eotaxin, granulocyte–colony-stimulating factor (G-CSF), stem cell factor (SCF), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) were obtained from R&D Systems (Minneapolis, MN). Assays were performed according to the manufacturer’s instructions. Briefly, culture supernatant samples and the corresponding cytokine standards were dispensed into the wells of 96-well plates. All samples were assayed without dilution except for the NHDF supernatants that were tested for IL-6 and IL-8. These samples were diluted 1:200 and 1:40 (v:v) in their appropriate assay diluents, respectively, so that the cytokine concentrations in the wells was below the maximum quantifiable amount for the individual assays. The wells were covered and allowed to incubate. The plates were washed thoroughly and an enzyme-conjugated detecting antibody was added to each well and allowed to incubate. Following another washing step, a substrate/chromogen solution was added into each well and the plates incubated in the dark at room temperature. A stop solution was added to the wells and the absorbance of each well was determined within 30 minutes. Absorbance was read at a wavelength of 450 nm using an EL800X microplate reader (Bio-Tek, Winooski, VT).

**Sample analysis.** In the experiments reported here, 12 individual wells containing keratinocytes and 12 containing fibroblasts were incubated with each test reagent (media, 50 μg of SS extract, 50 ng of LPS, and 50 μg of SS extract plus 50 ng of LPS). To provide enough volume of each sample to allow for the assay of 10 cytokines, supernatants from adjacent wells were pooled to provide six samples for each test reagent on each cell type that were then assayed. Data are reported as the mean ± SEM of the cytokine levels in the six pooled samples. Significance analysis was carried out using a Student’s two-tailed t-test to compare means.

**RESULTS**

**Time course and dose relationship.** In preliminary time course studies, it was determined that expression of most cytokines peaked about 24 hours post-challenge. Likewise, preliminary dose response studies showed significant release of cytokines with 50 or 100 μg of SS extract per well, and with 50 ng of LPS per well.

**Modulation of cytokines from NHEKs (Table 1).** IL-1 and IL-1ra. Cultured keratinocytes constitutively secreted small amounts of IL-1α and IL-1β (Table 1). Keratinocytes stimulated with LPS or with SS extract alone did not induce the elaboration of additional amounts of IL-1α. A small but significantly (P < 0.05) increased amount of IL-1β was secreted in response to the addition of SS extract in the absence or presence of LPS.

In contrast, keratinocytes constitutively produced large amounts of IL-1ra. Incubation with LPS alone had no effect on the production of IL-1ra. Keratinocytes stimulated with SS extract alone or along with LPS down-regulated production of IL-1ra by 23–31% compared with levels secreted in the presence of media or LPS alone, respectively.

IL-6. Cultured NHEKs constitutively secreted IL-6. Stimulation of these cells with SS extract induced a significant (P < 0.05) four-fold increase above the constitutive levels, while stimulation with LPS increased secretion by 38%. Co-incubation with LPS and SS extract nearly tripled (2.7 times) the amount secreted by stimulating with LPS alone (P < 0.05), reaching approximately the level observed with SS extract alone.

IL-8. Cultured keratinocytes also constitutively secreted IL-8. The SS extract induced a slight reduction in secretion of IL-8, while LPS induced an increase in secretion. Co-stimulation with both LPS and SS extract resulted in a significant (P < 0.05) 57% reduction in IL-8 secretion compared with the amount secreted by cells stimulated with LPS alone.

G-CSF. Keratinocytes did not constitutively secrete G-CSF nor did LPS induce secretion of this cytokine. Stimulation of keratinocytes with SS extract alone or in the presence of LPS induced elaboration of G-CSF.

VEGF. Keratinocytes constitutively secreted VEGF. This secretion was significantly (P < 0.05) up-regulated by 50% when the cells were stimulated with SS extract. LPS induced a 14% increase. LPS and SS extract combined induced a 31% increase in VEGF secretion over that induced by LPS alone; this level was the same as that induced by SS extract alone.

**Eotaxin, SCF, and TNF-α.** Keratinocytes did not constitutively secrete eotaxin, SCF, or TNF-α nor did they secret these cytokines when stimulated with SS extract, LPS, or with SS extract and LPS.

**TABLE 1**

Effect of *Sarcoptes scabiei* (SS) extract and *Escherichia coli* lipopolysaccharide (LPS) on cytokine production (pg/mL) by normal human epithelial keratinocytes*.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Detection limit</th>
<th>Media control</th>
<th>SS extract (50 μg)</th>
<th>LPS (50 ng)</th>
<th>SS extract (50 μg) + LPS (50 ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>1.0</td>
<td>83 ± 6</td>
<td>78 ± 3</td>
<td>81 ± 6</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.0</td>
<td>2.1 ± 0.4</td>
<td>4.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>30</td>
<td>2,767 ± 344</td>
<td>2,117 ± 78</td>
<td>2,912 ± 146</td>
<td>2,004 ± 134</td>
</tr>
<tr>
<td>IL-6</td>
<td>3.0</td>
<td>31 ± 6</td>
<td>121 ± 8</td>
<td>43 ± 6</td>
<td>117 ± 12</td>
</tr>
<tr>
<td>IL-8</td>
<td>10</td>
<td>88 ± 19</td>
<td>59 ± 5</td>
<td>111 ± 13</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>SCF</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VEGF</td>
<td>10</td>
<td>116 ± 20</td>
<td>174 ± 12</td>
<td>132 ± 11</td>
<td>173 ± 17</td>
</tr>
</tbody>
</table>

* Culture supernatants were collected 24 hours after stimulation. Values are the mean ± SEM. IL = interleukin; ra = receptor antagonist; ND = not detected; G-CSF = granulocyte–colony-stimulating factor; SCF = stem cell factor; TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor.
**Modulation of cytokines from NHDFs. IL-1 and IL-1ra.** Fibroblasts did not constitutively secrete IL-1α or IL-1β nor were these secreted when the cells were cultured in medium containing SS extract, LPS, or LPS + SS extract. Fibroblasts also did not constitutively produce IL-1ra. LPS induced a marked up-regulation of this cytokine. Incubation with SS extract alone, or in combination with LPS did not induce secretion above the constitutive or LPS-induced levels, respectively.

**IL-6 and IL-8.** Cultured fibroblasts did not constitutively produce IL-6 or IL-8. Stimulation of fibroblasts with SS extract induced an enormous secretion of both IL-6 and IL-8. LPS also induced secretion of these cytokines, but at levels that were significantly (P < 0.05) less than those induced by SS extract. Co-stimulation with LPS and SS extract resulted in levels that were similar to those of SS extract alone, but significantly (P < 0.05) greater than those observed with LPS alone.

**G-CSF.** The NHDFs did not constitutively secrete G-CSF. Cells stimulated with LPS alone showed a slight up-regulation of G-CSF secretion. Fibroblasts stimulated with SS extract or co-stimulated with SS extract plus LPS produced significantly (P < 0.05) more G-CSF.

**VEGF.** VEGF was the only cytokine constitutively secreted by cultured fibroblasts. Secretion of VEGF was significantly (P < 0.05) increased 4.5- and 2.6-fold when the cells were stimulated with SS extract alone or co-stimulated with SS extract and LPS, respectively. Stimulation with LPS alone did not significantly (P > 0.05) up-regulate production of VEGF.

**Eotaxin, SCF, and TNF-α.** Fibroblasts stimulated with SS extract or LPS and co-stimulated with SS extract and LPS did not produce eotaxin, SCF, and TNF-α nor were these compounds secreted constitutively.

**DISCUSSION**

* Sarcoptes scabiei* mites burrow in the lower stratum corneum of the skin and release substances that may produce effects on keratinocytes and fibroblasts that initiate and contribute to development of the inflammatory/immune reaction in the vicinity of the scabetic lesion.

In this study, we found that components in an SS extract induced marked increases in secretion of IL-6 and VEGF and slight increases in secretion of G-CSF from normal human epidermal keratinocytes. Components of the SS extract down-regulated keratinocyte secretion of IL-1ra, but did not influence the secretion of IL-1α or IL-1β. In comparison, components of the SS extract induced marked increases in secretion of IL-6, IL-8, G-CSF, and VEGF from normal human dermal fibroblasts. The significance of the secretion of this complex array of cytokines from these isolated effector cells *in vitro* and its relationship to the manifestation of the inflammatory and immune response to scabies *in vivo* is not entirely clear. *In vivo,* there is a complex interaction of many cell types and cytokines in the skin. However, based on the known functions of these cytokines, our findings do begin to elucidate how products from these mites may influence certain inflammatory/immune events in the host.

Interleukin-6 is known to have a diverse array of both proinflammatory and anti-inflammatory functions. It is known to stimulate proliferation of keratinocytes. One of the hallmarks of chronic scabies is hyperkeratosis. This is evident by the initial appearance of scaly skin and then the build-up of thick crusts (crusted scabies/Norwegian scabies) on the skin surface. Our previous histologic study of scabietic lesions showed an increase in keratinocytes undergoing mitosis in the basal layer of the epidermis. Up-regulation of IL-6 production by fibroblasts particularly, but also to a lesser extent by keratinocytes, that we observed would be consistent with these clinical and pathohistologic features of scabies.

Interleukin-6 likely plays other proinflammatory roles in the scabies reaction. It is known to increase vascular permeability, activate Th1 CD4+ cells to secrete IL-2 and promote their proliferation and differentiation, and activate Th2 CD4+ cells to produce IL-4, which drives antibody production. Hosts resistant to scabies have a strong Th1-like response while non-resistant hosts have a stronger Th2-like response (high antibody titer). CD4+ cells are a major component of the cell infiltrate in the scabietic lesion, possibly driven by IL-6. These findings have important implications in developing a vaccine to protect against scabies. Thus, further investigations are needed to more fully understand the significance of the up-regulation of IL-6 production in the host in response to scabies, its role in modulating the balance of the Th1 and Th2 response, and its role in developing protective immunity.

Our previous research showed that human skin equivalents (keratinocytes layered over a fibroblast/collagen matrix) se-
creted IL-1α and IL-1β in response to live scabies mites and their products. In contrast, our current study found that monocultures of human keratinocytes or fibroblasts stimulated separately with SS extract did not produce increased levels of IL-1α or IL-1β. Several possibilities exist that could explain these different results from the two systems: 1) a combination of factors (including communication among various cell types and their interactions with the matrix) in the more complex skin equivalents in vitro regulated production of this potent inducer of inflammation; 2) the dose (quantity or composition) of the SS molecules in extracts of this experiment were different from the live mites used to stimulate cultured skin-equivalent cells; 3) IL-1ra that was produced by the keratinocytes had an autocrine down-regulatory effect on the production of IL-1 in the monoculture cell system; and/or 4) enzymes present in the SS extract but not released or secreted in only small quantities by live mites degraded the IL-1 as it was produced. The fourth possibility is supported by a study by Mascia and others that found that an extract of the related house dust mite Dermatophagoides pteronyssinus induced a modest dose dependent release of both IL-1α and IL-1ra in cultured keratinocytes from healthy human donors, but the IL-1 was quickly degraded by enzymes in the extract. Enzymes in our scabies extract may have degraded the IL-1 if any IL-1 was actually secreted by the keratinocytes. A fifth possibility could involve the fact that the cells in the human skin equivalents came from human cadavers while the cells for this study were from normal healthy human donors (e.g., breast reduction surgery). Thus, the cells from dead versus living individuals may respond differently.

Keratinocytes, fibroblasts, monocytes, macrophages, bronchial epithelium, and neutrophils are known to produce IL-1ra. IL-1ra is a competitive inhibitor of IL-1 by binding to the IL-1α and IL-1β receptors on many target cells. It is a powerful inflammatory inhibitor, while IL-1 is a potent early mediator of inflammation and the immune response. Thus, the balance between IL-1 and IL-1ra influences the course of the inflammatory and overall immune responses. A significant finding in our study was that components in the SS extract induced a down-regulation of the constitutive secretion of IL-1ra by keratinocytes. The influence of down-regulation of IL-1ra from keratinocytes would be to up-regulate the influence of IL-1 from other cells such as Langerhans’ cells that are reported to migrate into the epidermis in the vicinity of a scabetic lesion. Therefore, our results suggest that IL-1ra may play a role in the up-regulation of the inflammatory response to scabies mites.

Cells with IL-1 receptors that can be blocked by IL-1ra are T cells, B cells, natural killer cells, vascular endothelial cells, macrophages, and neutrophils. Interleukin-1 stimulates activated T cells to synthesize IL-2 receptors. Activation of these IL-2 receptors by IL-2 from Th1 T helper cells promotes maturation of phagocytic cells. Interleukin-1 regulates the synthesis and expression of endothelial cell adhesion molecules such as E-selectin, P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 at the site of infection and thus influences the infiltration of inflammatory cells into the scabetic lesion. Enhancing the activity of IL-1 by reducing IL-1ra impacts many aspects of the overall inflammatory/immune response to scabies. In effect, it up-regulates induction of the inflammatory response.

One of the typical features of a primary scabies infestation in humans is that patients do not exhibit clinical manifestations or present with scabies until weeks after they have become infected because the inflammation develops slowly. This would not be consistent with an up-regulation of IL-1 and a down-regulation of IL-1ra, which together should promote development of inflammation. However, we observed production of large amounts of IL-6 from fibroblasts stimulated with SS extract. Interleukin-6 may actually have a down regulatory role early on by overriding the effects of the up-regulated IL-1 and down-regulated IL-1ra. This may explain the 6–8-week delay in developing clinical manifestations of this disease following a person contacting scabies. A primary infestation of a person likely results from very few mites entering the skin. It takes time for the few mites that penetrate the stratum corneum to reproduce and establish a larger population. This delay in the inflammatory/immune response would allow time for the few specimens that initiate the typical human infestation to become established.

Interleukin-8 is a potent chemoattractant for neutrophils by inducing adherence of these cells to vascular endothelial cells and their extravasation into tissues. Neutrophils are a dominant cell type in the dermal cellular infiltrate in scabietic lesions, more so for hosts that exhibit protective immunity to S. scabiei compared with those that do not. This observed accumulation of neutrophils in vivo may be due to chemotraction caused by up-regulated IL-8 production by fibroblasts in the dermis that is induced by products from scabies mites.

We found that fibroblasts also released large amounts of G-CSF. This cytokine promotes formation of neutrophils and monocytes that become dendritic cells. This is consistent with the appearance of large numbers of LCs in the epidermis and the strong neutrophil infiltrate observed in the scabietic lesion that was likely induced by IL-8.

When appropriately stimulated, keratinocytes and fibroblasts secrete VEGF. This is usually associated with wound healing. The stratum corneum where the mite burrows is generally dry and devoid of much extracellular fluid. VEGF causes increased vascular permeability and initiates inflammation. We have observed that scabietic lesions show some edema and that fluid seeps into the burrow in front of the mite. This fluid is thought to be the main source of nutrition and water for the mites. These observations suggested that the mites could induce production of VEGF by keratinocytes or fibroblasts. Consistent with these observations, we found that both keratinocytes and fibroblasts secreted VEGF in response to SS extract. Nutritionally, this would be beneficial to survival of the mites and help them maintain water balance.

The complex immunology that occurs in the epidermis and dermis of the skin that regulates the inflammatory/immune response to scabies is not well understood. Our current study suggests that components in scabies extract modulate the inflammatory/immune reaction during scabies infestation. It is a complex interaction and it is as yet difficult to dissect out specific up-regulated and down-regulated events that direct the course in the host reaction. Much more study along these lines is required to fully understand the host-parasite interaction.

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