Cytokine-Stimulated Human Dermal Microvascular Endothelial Cells Produce Interleukin 6—Inhibition by Hydrocortisone, Dexamethasone, and Calcitriol

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The effects of lipopolysaccharide (LPS), recombinant human tumor necrosis factor-α (TNF), recombinant human interleukin 1-beta (IL-1β), and interferon-γ (IFN-γ) on IL-6 production were determined by enzyme-linked immunosorbent assay (ELISA) and by Northern blot analysis in cultured human dermal microvascular endothelial cells (HDMEC). Unstimulated HDMEC did not produce significant amounts of IL-6, whereas lipopolysaccharide (LPS), TNF, and IL-1β were potent inducers of HDMEC-derived IL-6 production. Treatment with IFN-γ had no effect. IL-1β stimulation resulted in pronounced IL-6 production after 4 h, followed by complete downregulation at the transcriptional level after 24 h. In contrast, LPS and TNF induced prolonged stimulation of IL-6 production by HDMEC as IL-6 mRNA transcripts were still detected after 24 h treatment and IL-6 protein was markedly increased at this timepoint.

Interleukin 6 (IL-6) is a multifunctional pro-inflammatory cytokine produced by a variety of cell types, such as lymphocytes, monocytes, fibroblasts, and others [1–5]. It has also been described as recombinant human interferon-β (IFN-β) [6], B cell-stimulating factor [7], hybridoma/plasmacytoma growth factor [8], and hepatocyte-stimulating factor [9], indicating its pleiotropic effects and suggesting a major role in immunologic and inflammatory reactions [10]. Recently, it has been reported that IL-6 is overexpressed in psoriatic skin lesions, and elevated IL-6 levels have been detected in the plasma of psoriatic patients [11,12]. Furthermore, an increased IL-6 production by monocytes, keratinocytes [12], and fibroblasts [13] has been described in patients with psoriasis. As activation of the dermal endothelium is one of the earliest events in the development of psoriatic lesions [14,15], human dermal microvascular endothelial cells (HDMEC) may well contribute to the pathogenesis of psoriasis by producing pro-inflammatory cytokines. Previous studies have shown that stimulated human umbilical vein endothelial cells (HUVEC) in vitro also produce significant amounts of IL-6 [16–19]. However, microvascular endothelium differs from large vessel endothelium by several properties [20,21], and it has been recently shown by us and others that HDMEC clearly differ from HUVEC with regard to their expression of cell adhesion molecules [22], their proliferative response upon cytokine stimulation [23–25], and their response to histamine treatment [26]. Therefore, the cultivation of HDMEC in vitro [27,28] seems to be a more appropriate model for studies on the contribution of dermal endothelial cells to inflammatory skin disorders. In the present study we investigated whether

HDMEC: human dermal microvascular endothelial cells
HUVEC: human umbilical vein endothelial cells
IFN-γ: recombinant human interferon-γ
IL-α: recombinant human interleukin 1-α
IL-1β: recombinant human interleukin 1-β
IL-6: interleukin 6
LPS: lipopolysaccharide
MOPS: 3-(N-morpholino)propanesulfonic acid
PBS: phosphate-buffered saline
SD: standard deviation of the mean
SDS: sodium dodecyl sulfate
TNF: recombinant human tumor necrosis factor-alpha

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Abbreviations:
EBM: endothelial cell basal medium
EC: endothelial cells
EGF: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
FCS: fetal calf serum
GM-CSF: recombinant human granulocyte/macrophage colony-stimulating factor

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dermal endothelium may be a major source for IL-6 after stimulation with the pro-inflammatory cytokines IL-1β and TNF in vitro, and therefore actively participate in the pathogenesis of inflammatory skin diseases such as psoriasis. Furthermore, the antiprosthetic drugs hydrocortisone, dexamethasone, calcitriol, acitretin, and cyclosporine A were tested for their ability to inhibit the HDMEC-derived IL-6 production in vitro.

MATERIALS AND METHODS

Endothelial Cell Cultures  Cultures of HDMEC were established as previously described [25,29]. In short, human foreskins obtained by routine circumcisions of neonatal or children were cut into small pieces, and after repeated washes with phosphate-buffered saline (PBS) containing 400 U/ml penicillin, 400 U/ml streptomycin, and 0.5 μg amphotericin B (Clonetics, San Diego, CA), the tissue pieces were incubated overnight in 0.25% trypsin in PBS (Biochrom KG, Berlin, Germany) at 4°C. After removal of the epidermis, the dermal parts were rinsed in Iscove’s medium supplemented with 10% fetal calf serum (FCS, Biochrom). Microvascular fragments were released into the medium by mechanical treatment of the dermal tissue with a scalpel blade. After passing through a sterile 100-μm nylon mesh (Reichert, Heidelberg, FRG) the cell suspension was centrifuged at 200 × g for 10 min, and the cells were resuspended in Iscove’s medium containing 10% FCS. Endothelial cells (EC) were then purified by continuous Percoll (Sigma, St. Louis, CA) density gradient centrifugation at 400 × g for 15 min as described [25,29], washed in Iscove’s medium, and resuspended in endothelial cell basal medium (EBM, Clonetics), supplemented with 10% FCS, 10 ng/ml epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, and antibiotics (Clonetics). Endothelial cells were then plated at a seeding density of 2 × 10⁴ cells/cm² on 80-mm fibronectin-coated plastic Petri dishes (Costar, Cambridge, MA) and incubated at 37°C in 100% humidity in an atmosphere of 95% air, 5% CO₂. The medium was replaced every second day, and the cells were passaged before confluency. The endothelial origin of the cultured cells was confirmed by expression of Factor VIII-related antigen and by staining with the lectin Ulex europaeus-1 [29]. For all investigations confluent second-passage HDMEC were used.

Treatment with Cytokines and Antiprosthetic Drugs  Second-passage HDMEC cultures were treated with 10 μg/ml lipopolysaccharide from Salmonella minnesota (LPS, Sigma), TNF-α (10–1000 U/ml, BASF-Knoll, Mannheim, Germany, specific activity 6.63 × 10⁶ U/mg), IFN-γ (10–1000 U/ml, Bioferon, Laupheim, Germany, specific activity 2.29 × 10⁸ U/mg), or IL-1β (10–1000 U/ml, British Biotechnology, Oxford, UK, specific activity 5 × 10⁸ U/mg) in EBM without hydrocortisone or FCS for 4 or 24 h. Cultures incubated in EBM alone were used as negative controls. As positive controls for the activity of the examined cytokines, the induction of intercellular adhesion molecule-1 (ICAM-1) by TNF, IL-1β, and IFN-γ was assessed by immunocytochemistry as described [24]. Supernatants were then assayed for IL-6 production by ELISA, and total RNA was extracted for IL-6 mRNA detection by Northern blot analysis as described below.

In further experiments, confluent second-passage HDMEC were incubated for 24 h with EBM, TNF (1000 U/ml), or IL-1β (1000 U/ml) either alone or in combination with hydrocortisone (10⁻⁵–10⁻¹⁰ M, Sigma), dexamethasone (10⁻⁵–10⁻¹² M, Sigma), acitretin (10⁻⁵–10⁻⁷ M, Hoffman-LaRoche, Basel, Switzerland), cyclosporine A (10⁻⁵–10⁻⁸ M, Sandoz, Basel, Switzerland) or the vitamin D₃ analogue calcitriol (10⁻⁵–10⁻⁸ M, Hoffman-LaRoche), and supernatants were assayed for IL-6 production by ELISA. Three independent experiments were carried out, and the results were expressed as percent of the corresponding TNF or IL-1β–treated controls. All results are given as mean ± standard deviation (SD) of the mean. Statistical significance was calculated using the two-sided Student t test.

RNA Isolation and Northern Blot Analysis of IL-6 Transcripts  Total cellular RNA was isolated using the acid guanidi-
Figure 1. IL-6 production by HDMEC treated with EBM (cont), LPS, IL-1β, TNF, or IFN-γ for 4 h detected by ELISA and Northern blot analysis. Results represent one of three comparable experiments.

LPS for 24 h (Fig 2). Again, IFN-γ failed to induce IL-6 production by HDMEC, as detected by ELISA.

Northern blot analysis of IL-6 mRNA after 24 h of treatment did not reveal any detectable message in HDMEC treated with IL-1β (Fig 2). According to the significantly elevated IL-6 protein levels in LPS- or TNF-treated cultures after 24 h, IL-6 mRNA transcripts also were still clearly detectable in these cultures after 24 h. However, the intensity of the signals was reduced, as compared to the results after 4 h stimulation (Fig 2). HDMEC cultures treated with EBM alone or with IFN-γ did not reveal IL-6 transcripts after 24 h.

Effects of Antipsoriatic Drugs on Cytokine-Induced IL-6 Production

In order to investigate whether the effects of antipsoriatic drugs may be partly mediated by inhibition of the cytokine-induced IL-6 production by dermal endothelium, HDMEC were treated with IL-1β or TNF either alone or in combination with hydrocortisone, dexamethasone, acitretin, cyclosporine A, or calcitriol for 24 h. Although HDMEC cultures treated with IL-1β or TNF alone produced high amounts of IL-6, as detected by ELISA, combination with corticosteroids markedly inhibited this induction in a dose-dependent manner (Fig 3). Dexamethasone proved to be a more potent inhibitor as compared to hydrocortisone. In contrast, acitretin and cyclosporine A did not influence the IL-1β-induced IL-6 production, whereas calcitriol dose-dependently inhibited this stimulation (Fig 4).

Combination of TNF with antipsoriatic drugs confirmed these results. Again, hydrocortisone and dexamethasone reduced the cytokine-stimulated IL-6 production, but the inhibition was less pronounced as compared to IL-1β-treated HDMEC (Fig 5). Acitretin or cyclosporine A were not effective (Fig 6), whereas the combination of TNF with calcitriol resulted in a reduction of HDMEC-derived IL-6 release. No significant IL-6 production was found in the supernatants of control cultures that received hydrocortisone, dexamethasone, acitretin, cyclosporine A, calcitriol, or EBM alone (data not shown).

DISCUSSION

The present investigation clearly shows that HDMEC are a major source of the pro-inflammatory cytokine IL-6 when treated with...
ments using the same cytokine concentrations that IFN-γ was a strong inducer of ICAM-1 expression on HDMEC (data not shown). A moderate [17] or strong [16] induction of IL-6 by IFN-γ has been reported in HUVEC, and pre-incubation or co-incubation of IFN-γ with TNF, IL-1β, or LPS resulted in augmented production of IL-6 [34]. Despite these inhomogeneous effects in HUVEC, our data suggest a further difference between microvascular and large vessel EC regarding their cytokine response, in addition to the reported differences in the expression of ICAM-1 [22] and VCAM-1 [35], melanoma cell adhesion [36], their proliferative response upon cytokine stimuli [23–25], and their responses to histamine treatment [26].

The present results demonstrate that, beside other cell types such as T lymphocytes or macrophages, dermal endothelium is a major source of the pro-inflammatory cytokine IL-6. Recently, elevated IL-6 levels have been found in the plasma of psoriatic patients, and increased IL-6 production by monocytes, keratinocytes, and fibroblasts has been reported in patients with psoriasis [11–13]. Furthermore, increased susceptibility to the growth-promoting effects of IL-6 has been found in psoriatic keratinocytes in vitro, as compared to normal keratinocytes [37]. As activation of the dermal endothelium is an early event in the development of psoriatic lesions [14,15], HDMEC-derived IL-6 may play a keyrole in the pathogenesis of psoriasis.

Based on this finding we examined the influence of several anti-psoriatic drugs on IL-6 production and found that hydrocortisone and dexamethasone exerted dose-dependent inhibitory effects on IL-6 production by HDMEC. The observed inhibition was independent of the source of stimulation as it was detected after IL-1β as well as TNF treatment. The marked inhibitory potency of steroids on HDMEC IL-6 production is in accordance to the findings in fibroblasts and HUVEC, where cortisol and dexamethasone were also potent inhibitors of IL-1β–induced IL-6 production [38]. As there is a glucocorticoid-responsive element located in the IL-6 gene region and as IL-6 gene expression can be shut off at the transcriptional level in response to glucocorticoids, this might be the regulatory site [38,39]. The vitamin D₃ analogue calcitriol also inhibited HDMEC IL-6 production, but was less effective compared to the used corticosteroids. Vitamin D₃ compounds such as calcitriol mediate their effects by binding to a specific nuclear receptor that shares homology with other steroid receptors [40,41] and may therefore share common regulatory pathways. Beside the anti-proliferative effect of calcitriol on keratinocytes [42], the inhibition of HDMEC IL-6 production may also contribute to its beneficial action for treating psoriasis.

In contrast, acitretin and cyclosporine A did not influence HDMEC IL-6 production, suggesting other mechanisms of action in psoriasis. Both substances are known to inhibit keratinocyte proliferation [43–46], and cyclosporine A is a potent immunosuppressive agent, decreasing the number of activated lymphocytes and neutrophils as well as IL-2 production in psoriasis [47–49].

In conclusion, the present study demonstrates that activated human dermal microvascular endothelial cells are a major source of the pro-inflammatory cytokine IL-6 and, therefore, may actively contribute to the pathogenesis of inflammatory skin disorders. As IL-6 seems to play a key role in the pathogenesis of psoriasis, studies on the modulation of HDMEC IL-6 release may provide further insight into the cytokine regulation of early psoriatic alterations, as well as into the mechanisms of action of antipsoriatic therapy.

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**Figure 5.** IL-6 production by HDMEC treated with TNF (1000 U/ml) either alone or in combination with hydrocortisone (10⁻⁵ – 10⁻¹¹ M, circles) or dexamethasone (10⁻⁵ – 10⁻¹¹ M, squares) for 24 h. Results are expressed as percent of TNF-treated controls ± SD (● p < 0.05; ** p < 0.01; *** p < 0.001; versus control).

**Figure 6.** IL-6 production by HDMEC treated with TNF (1000 U/ml) either alone or in combination with calcitriol (10⁻⁵ – 10⁻⁸ M), acitretin (10⁻⁵ – 10⁻⁷ M), or cyclosporine A (10⁻⁵ – 10⁻⁷ M) for 24 h. Results are expressed as percent of TNF-treated controls ± SD (● p < 0.05; versus control).

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