A Proinflammatory Activity of Interleukin 8 in Human Skin: Expression of the Inducible Nitric Oxide Synthase in Psoriatic Lesions and Cultured Keratinocytes

By Daniela Bruch-Gerharz,* Karin Fehsel,* Christoph Suschek,* Guenter Michel,* Thomas Ruzicka,* and Victoria Kolb-Bachofen*

From the *Research Group Immunobiology, Biomedical Research Center; and †Department of Dermatology, Heinrich-Heine-University of Duesseldorf, D-40001 Duesseldorf, Germany

Summary

Psoriasis is a common chronic skin disease mediated by cellular immune mechanisms and characterized by an intense neutrophil cell infiltrate and proliferative activation of epidermal keratinocytes. We have previously described the expression of the inducible nitric oxide synthase (iNOS) in epidermal keratinocytes of psoriatic skin lesions. In this study, the role of iNOS in psoriatic inflammation was explored ex vivo in psoriatic skin biopsies and in vitro in primary cultures of human keratinocytes. Messenger RNA for the iNOS enzyme (iNOS mRNA) was detected by reverse transcriptase polymerase chain reaction in skin biopsies from patients with psoriasis, but not in skin specimens from patients with atopic eczema or from healthy volunteers. As demonstrated by in situ hybridization and immunohistochemistry, expression of iNOS mRNA and its gene product was localized to the epidermal keratinocytes of psoriatic skin lesions. In situ hybridization further revealed a complete colocalization of mRNA expression for iNOS with interleukin (IL) 8 receptor–specific mRNA either in the basal germinative cell layer or at focal sites of ongoing neutrophil inflammation in suprabasal cell layers. Because psoriatic keratinocytes have previously been shown to express mRNA transcripts for IL-8, it seemed reasonable to hypothesize that iNOS expression could be induced in an autocrine loop by IL-8. This hypothesis was substantiated by our in vitro experiments showing that a combination of IL-8 and interferon γ induces the expression of iNOS–specific mRNA and of the functional enzyme in cultured human keratinocytes. These results suggest an important role for iNOS in concert with IL-8 and its receptor early during the formation of psoriatic lesions.
each of which potentially contributes to epidermal activation (2, 4). Most recently, it became evident that IL-8 appears to be pivotal in the pathogenesis of psoriatic lesions, responsible for prominent leukocytic infiltration as well as keratinocyte hyperproliferation (12, 13). In epidermal keratinocytes of psoriatic skin lesions we demonstrated increased expression of IL-8 and its receptor (IL-8R) (13). Furthermore, we identified the epidermal IL-8R as the molecular target for the immunomodulatory antipsoriatic drug FK 506 (tacrolimus) as well as other antipsoriatic agents (14, 15).

It is currently well established that the cytokine-inducible NOS is involved in diverse physiologic and pathophysiologic processes, including host immune defense, immunoregulation, and tissue destruction in autoimmune diseases (16, 17). Investigations on its role and expression in common skin diseases, however, are lacking. This report demonstrates that mRNA transcripts of iNOS, expressed by epidermal keratinocytes in psoriatic skin lesions, are colocalized with mRNA transcripts of the epidermal IL-8R. Moreover, we here show for the first time that IL-8 is a potent costimulatory inducer for iNOS expression in cultured human keratinocytes, providing a mechanism to link induction of iNOS with the characteristic neutrophil inflammation in psoriasis.

Materials and Methods

Selection and Preparation of Skin Sections

Fresh keratoma biopsies were taken from lesional and nonlesional skin of 15 patients with untreated, longstanding psoriasis and, for control purposes, from 10 patients with atopic eczema and 10 healthy volunteers. Skin biopsies were flash frozen in liquid nitrogen and used for either reverse transcriptase (RT) PCR, in situ hybridization, or indirect immunohistochemistry.

Cell Stimulation and Culture

Cell Culture Reagents. Complete medium consisted of Keratinocyte-SFM (Gibco Laboratories, Grand Island, NY) supplemented with 2.5 ng/ml epidermal growth factor, 25 μg/ml bovine pituitary extract, 70 U/ml penicillin, and 70 μg/ml streptomycin. Homogeneous IL-8 was obtained from R & D Systems (Abingdon, UK). Recombinant human IFN-γ, TNF-α, and IL-1β were purchased from HBT (Leiden, Netherlands).

Cell Purification. Primary keratinocytes were prepared from reduction mammoplasty specimens essentially as described (3). Cells were grown in vitro on plastic substrates under standard culture conditions. Primary cultures consisted of pure keratinocyte cultures also demonstrated by anticytokeratin immunocytochemistry.

Cell Stimulation. For stimulation of iNOS expression, primary human keratinocytes were cultured in Keratinocyte-SFM without epidermal growth factor and bovine pituitary extract. Confluent monolayers were incubated for 24 h in the presence or absence of IL-8, IFN-γ, TNF-α, and/or IL-1β. The capacity of these cytokines to induce iNOS expression was evaluated using RT-PCR amplification, in situ hybridization, indirect immunocytochemistry, and a colorimetric nitrite assay to quantify nitrite accumulation in the cell culture medium. Experiments were performed with cells in either their second or third passage in vitro.

RT-PCR Analysis

Freshly harvested epidermal cells or tissues were flash frozen in liquid nitrogen. Total RNA extraction was performed using guanidinium thiocyanate as described (13). First strand cDNA was prepared from 1 μg of RNA using Avian moloney leukemia virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) under the conditions suggested by the supplier. After reverse transcription, amplification was carried out by PCR using Taq polymerase (Gibco Laboratories) with 15 mM MgCl₂. PCR was performed with primers specific for iNOS (5'-ATGCCAGATGCCCAGCATCAGA-3' and 5'-TTTCCAGGCCCCATTCC-TGC-3'), yielding a 394-bp product; and for glyceraldehyde-3-phosphate-dehydrogenase (G3PDH; 5'-ACAGTTCCATGCCATCAGGCGC-3' and 5'-GCCTGTCTTCACACCTTCTTG-3') to yield a 228-bp product. Cycle times were 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 35 cycles. 20% of the iNOS reaction was combined with 5% of the G3PDH reaction and analyzed for the presence or absence of the iNOS species by electrophoresis on a 2% agarose gel.

In Situ Hybridization Studies

Preparation of Digoxigenin-labeled mRNA Probes. iNOS cDNA from mouse macrophage was a generous gift from Drs. Carl Nathan and Qiao-wen Xie (Cornell University Medical College, New York, NY). cDNA of IL-8R type A was kindly provided by Dr. William Wood (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Both DNA probes were subcloned in a transcription vector as previously described (18). After linearization of plasmid DNA with appropriate restriction enzymes, labeled antisense and sense riboprobes were synthesized by in vitro transcription of cDNA, cloned downstream of SP6 or T7 promoters, with SP6 or T7 RNA polymerase using digoxigenin-labeled UDP as substrate (Boehringer Mannheim).

Hybridization Procedure. The protocol described by Letch et al. (18) was used with minor alterations. In brief, 7-μm cryostat sections or cultured human keratinocytes were hybridized with digoxigenin-labeled antisense and sense riboprobes for iNOS or IL-8R. After posthybridization high-stringency washing procedures, slides were incubated with an alkaline phosphatase-labeled antidigoxigenin Ab (Boehringer Mannheim). Visualization of the probe hybridization side was performed using 5-bromo-4-chloro-3-indolyl phosphate toluidine and nitroblue tetrazolium salt as chromogenic substrate (Boehringer Mannheim). No positive hybridization signals were observed in any specimens treated with the sense riboprobes.

Immunohistochemical Analysis of iNOS Protein

The immunohistochemical procedures followed methods described previously (5) and were performed on 7-μm frozen sections of keratoma biopsies or cultured human keratinocytes. A mouse mAb to macrophage iNOS (Transduction Laboratories, Lexington, KY) was used to assess expression of iNOS protein. For negative controls, the first mAb was either omitted or replaced by an irrelevant isotype-matched reagent. These experiments consistently yielded negative results. Because it has recently been observed that the iNOS Ab may cross-react with the constitutive NOS proteins (19), serial sections were also stained with a polyclonal anti-neuronal antisera and a monoclonal anti-endothelial Ab (both from Transduction Laboratories) to test the specificity of the iNOS mAb. None of these latter Abs resulted in staining of epidermal keratinocytes.
Nitrite Assay

NO synthesis in primary cultures of human keratinocytes was determined by measuring the accumulation of nitrite (NO$_2^-$), a stable end product of NO metabolism, using a modified Griess reaction as described earlier (19).

Results and Discussion

The specific expression of iNOS mRNA ex vivo was determined in keratoma biopsies from patients with psoriasis or atopic eczema and from healthy volunteers using RT-PCR amplification. iNOS mRNA transcripts were demonstrated in all keratoma biopsies (n = 15) from lesional psoriatic skin and in 5 out of 15 of the corresponding nonlesional skin biopsies (Fig. 1 A). The strong signal for iNOS mRNA observed in these nonlesional skin specimens appears to be due to preclinical inflammatory skin alterations. In contrast, all keratoma biopsies of atopic eczema (n = 10) were devoid of any iNOS message, as were all skin specimens from healthy volunteers (n = 10) (Fig. 1, B and C).

To determine the localization of iNOS mRNA transcripts in psoriatic skin lesions, in situ hybridization was performed using cryostat sections of the same biopsies (n = 5 for each of the three groups). Specific signals for iNOS mRNA were detected in keratinocytes of the epidermal compartment of psoriatic skin biopsies showing two essentially different localization patterns. In two out of five biopsies, keratinocytes of the highly proliferative basal epidermal layer yielded a strong and specific signal for iNOS mRNA located in a bandlike pattern, where the hybridization signal was preferentially observed at the tips of the rete ridges (Fig. 2 A). In three out of five lesional skin specimens, however, epidermal keratinocytes showed a focal pattern of expression in the suprabasal cell layers, often closely associated with focal clusters of infiltrating inflammatory cells of neutrophil morphology (Fig. 2 C). In two out of five nonlesional psoriatic skin specimens, abundant signals were detected in the basal epidermal layer (Fig. 2 E), again suggesting a potential role of iNOS in the early phase of tissue inflammation in psoriasis.

Immunohistochemical staining of psoriatic skin specimens (n = 10) also localized the iNOS to the epidermal keratinocytes. Identical to in situ hybridization patterns, keratinocytes that stained for iNOS were either localized in the basal epidermal layer (Fig. 2 F) of lesional or nonlesional skin, respectively, or were focally clustered at sites of ongoing neutrophil inflammation in suprabasal cell layers (Fig. 2 G). In contrast, no epidermal iNOS mRNA or protein was detected in any of the skin specimens from patients with atopic eczema nor from healthy volunteers (data not shown).

IL-8 is known to play a critical role in neutrophil chemotaxis (12, 13), and mRNA transcripts for IL-8 or IL-8R and IL-8 immunoreactivity were found either focally clustered in suprabasal keratinocytes associated with infiltrating neutrophils or confined to basal epidermal keratinocytes of psoriatic skin lesions (12, 20). Because we have shown increased expression of mRNA transcripts for IL-8 and its receptor in epidermal keratinocytes of psoriasis using RT-PCR amplification (13), we now analyzed the localization of IL-8R–expressing cells in serial sections of the same psoriatic skin specimens using in situ hybridization. In complete accordance with the expression pattern observed for iNOS gene expression, we found IL-8R mRNA transcripts to be colocalized with iNOS mRNA transcripts either in the basal epidermal compartment or in the suprabasal cell layers at sites of ongoing neutrophil inflammation (Fig. 2, B and D). The simultaneous expression of both iNOS and IL-8R gene in psoriatic epidermal keratinocytes suggests a paracrine and/or autocrine pathway involved in
local leukocyte recruitment as well as keratinocyte activation.

Earlier studies have shown that the iNOS gene can be stimulated in a number of mammalian cells by proinflammatory cytokines, notably IFN-γ, TNF-α, and IL-1β (10, 11). A direct interaction, however, between iNOS gene expression and the cytokines known to be involved in psoriatic inflammation (2, 20) has not yet been demonstrated for primary human keratinocytes. To examine whether epidermal keratinocytes can be induced to express iNOS
Whereas none of these cytokines was active when added as by signals present in a psoriatic microenvironment, we experiments performed in duplicate. detection methods (Fig. 5). Increased nitrite formation was the response profiles seen with iNOS mRNA and protein detected in unchallenged keratinocytes. Nitrite accumulation in the cell culture media was found to exactly parallel the response profiles seen with iNOS mRNA and protein detection methods (Fig. 5). Increased nitrite formation was not detected in the presence of 0.5 mM NG-monomethyl-L-arginine. These findings introduce iNOS as a molecular signal present in the inflammatory response of epidermal keratinocytes and extend our previous efforts to unravel the molecular pathophysiology of inflammation in psoriasis.

Moreover, our studies establish a unique profile of cytokine responsiveness in human keratinocytes, because iNOS expression could only be induced by synergistic combinations of IL-8 plus IFN-γ or TNF-α plus IL-1β. This profile for NOS induction clearly distinguishes human keratinocytes from other cells and tissues in which inducibility is also conferred by single activators or other cytokine combinations. Thus, expression of iNOS in human hepatocytes—of epithelial cell origin also—is known to be controlled at the level of transcription by IL-1β alone or in combination with IFN-γ (8), which is ineffective in keratinocytes. The observation of iNOS expression in psoriatic but not in atopic skin specimens demonstrates a key role for the local cytokine profile in the regulation of iNOS expression in vivo.

Our contention that iNOS is involved in the pathogenesis of psoriasis is further supported by the antiinflammatory effects of retinoids in the treatment of psoriasis, which have been shown to inhibit iNOS gene transcription (21).

Figure 5. Nitrite production by cytokine-challenged human keratinocyte cultures. Primary human keratinocytes were cultured in the presence or absence of 10 nM IL-8, 1,000 U/ml IFN-γ, 1,000 U/ml TNF-α, and 1,000 U/ml IL-1β. After 24 h of incubation, nitrite production in the culture media was determined spectrophotometrically by a modified Griess reaction, and the presented results are the mean ± SD of three experiments performed in duplicate.
Further elucidation of the molecular signals involved in the regulatory and effector mechanisms of iNOS expression in epidermal keratinocytes may give insights into the pathogenesis of psoriasis. Modulation of iNOS expression could provide a therapeutic aim in psoriasis and other immune-mediated skin diseases.

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Address correspondence to Dr. Daniela Bruch-Gerharz, Research Group Immunology, Building 14.80, Heinrich-Heine-University, P.O. Box 101007, D-40001 Duesseldorf, Germany.

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