Critical Role of HIF-1α in Keratinocyte Defense against Bacterial Infection

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Skin, the first barrier against invading microorganisms, is hypoxic, even under baseline conditions. The transcription factor hypoxia-inducible factor-1α (HIF-1α, the principal regulator of cellular adaptation to low oxygen, is strongly expressed in skin epithelium. HIF-1α is now understood to play a key role in the bactericidal capacity of phagocytic cells such as macrophages and neutrophils. In the skin, keratinocytes provide a direct antibacterial activity through production of antimicrobial peptides, including cathelicidin. Here, we generate mice with a keratinocyte-specific deletion of HIF-1α and examine effects on intrinsic skin immunity. Keratinocyte HIF-1α is seen to provide protection against necrotic skin lesions produced by the pathogen group A *Streptococcus*. RNA interference studies reveal that HIF-1α regulation of keratinocyte cathelicidin production is critical to their antibacterial function.

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INTRODUCTION

The skin not only serves as a mechanical barrier to infection, but also participates in innate immune defense through production of cationic antimicrobial peptides capable of inactivating many microorganisms (Boukamp *et al.*, 1988; Ganz, 2002; Braff and Gallo, 2006). Cathelicidins are a diverse family of antimicrobial peptides with broad-spectrum activities; a single gene-encoded cathelicidin is expressed by humans (LL-37) and mice (mCRAMP) (Tomasinsig and Zanetti, 2005). Mice lacking mCRAMP are hyper-susceptible to epithelial infection (Nizet *et al.*, 2001), whereas those overexpressing cathelicidin are more resistant (Lee *et al.*, 2005), together demonstrating the critical role of cathelicidin in skin innate immunity. While cathelicidins may be derived from several cell types present in the skin, including keratinocytes (Frohm *et al.*, 1997; Sorensen *et al.*, 2003), neutrophils (Turner *et al.*, 1998) and mast cells (Di Nardo *et al.*, 2003), a direct contribution of keratinocyte cathelicidin production to antibacterial immunity has been proven (Braff *et al.*, 2005).

Both human and mouse skin are known to be hypoxic, with baseline oxygen levels ranging between 1.5 and 5.0% (Evans and Naylor, 1967; Stewart *et al.*, 1982). Apart from stratum corneum, oxygen is consumed in all the layers of the epidermis and dermis. The oxygen demand is partially satisfied by the blood in the dermis. In contrast, the epidermis has no vasculature. Bedogni *et al.* (2005) recently reported that normal human and mouse skin present hypoxic regions as indicated by positive staining of the hypoxia markers carbonic anhydrase IX and 2-nitroimidazole EF5 (EF5). These markers correlate with glucose transporter-1 staining, a well-known HIF1α direct target gene. These observations suggest the skin microenvironment would support the activity of the transcriptional regulator hypoxia-inducible factor-1α (HIF-1α), known to orchestrate cellular adaptation to low-oxygen environments. HIF-1 is a heterodimer whose expression is regulated post-translationally. In the presence of oxygen, HIF-1α is hydroxylated by iron-dependent prolyl hydroxylases and is then degraded through the ubiquitin–proteasome pathway via its interaction with von Hippel–Landau. Under hypoxia, prolyl hydroxylase activity is inhibited. HIF-1α then accumulates and translocates into nucleus, where it binds to ARNT (aryl hydrocarbon receptor nuclear translocator)/HIF-1β, which is constitutively expressed. The heterodimer HIF-1 binds to the hypoxic response elements of target gene-regulatory sequences, resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress (Poellinger and Johnson, 2004). Recently, we uncovered a role for HIF-1α in mediating the bactericidal capacities of immune cells, including neutrophils and macrophages (Cramer *et al.*, 2003; Peyssonnaux *et al.*, 2005). Here, we

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Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; cfu, colony-forming units; EF5, 2-nitroimidazole EF5; GAS, group A Streptococcus*; hCAP-18, human cathelicidin antimicrobial protein-18; HIF-1α, hypoxia-inducible factor-1α; OCT, Tissue-Tek compound*; siRNA, small interfering RNA

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hypothesize that HIF-1α expression in keratinocytes could be critical to the function of these specialized epithelial cells in skin innate immunity. To address this question, we generate a mouse harboring a targeted deletion of HIF-1α in keratinocytes. Infectious challenge of these mice is coupled with *in vitro* tissue culture studies to examine the role of HIF-1α in skin antibacterial defense.

RESULTS AND DISCUSSION

Normal mouse skin is hypoxic, as evidenced by strong positive immunostaining for the Hypoxyprobe™-1 reagent (Figure 1a). This marker correlates to pronounced immunostaining for transcriptional regulator HIF-1α within the epithelial keratinocyte layer (Figure 1a). The constitutive expression of HIF-1α in normal skin suggests its role in regulating key biological processes such as the ability of skin to defend against invasive microorganisms. Since keratinocytes are the major cell type of the epidermis, we generated a mouse harboring keratinocyte-specific inactivation of HIF-1α to directly examine the role of the transcriptional regulator in skin innate immunity *in vivo*. Deletion efficiency in epidermis was ~45% as determined by quantitative PCR (data not shown). As keratinocytes account for 90% of the epidermis, we can estimate that ~50% of keratinocytes are HIF-deficient. As expected, deletion of HIF does not prevent the occurence of hypoxia. (Figure 1b).

A well-established model of group A *Streptococcus* (GAS)-induced necrotizing skin infection (Nizet *et al.*, 2001; Datta *et al.*, 2005) was utilized. An inoculum of 1×10^7 colony-forming units (cfu) of GAS was injected subcutaneously into a shaved area on the flank of wild-type (WT) or HIF-1α$^{flox/flox}$/K14cre (HIF-1α$^{-/-}$) male littermates, and the progression of infection followed over 9 days. Mice with a tissue-specific deletion of HIF-1α in their keratinocytes developed significantly larger necrotic skin lesions than WT controls (Figure 2a). We next asked whether keratinocyte production of HIF-1α was important in limiting the ability of the GAS to replicate within the necrotic skin tissues. Mice were killed at 96 hours post-inoculation, and quantitative bacterial cultures performed on the skin ulcer (or site of inoculation if no ulcer developed) (Figure 2b). Approximately threefold greater quantities of GAS were present in the skin biopsies of HIF-1α-null mice relative to WT mice, indicating the presence of HIF-1α transcriptional control in keratinocytes to be important in limiting the extent of necrotic tissue.

An important component of innate immune defense in mammals is the cathelicidin family of antimicrobial peptides

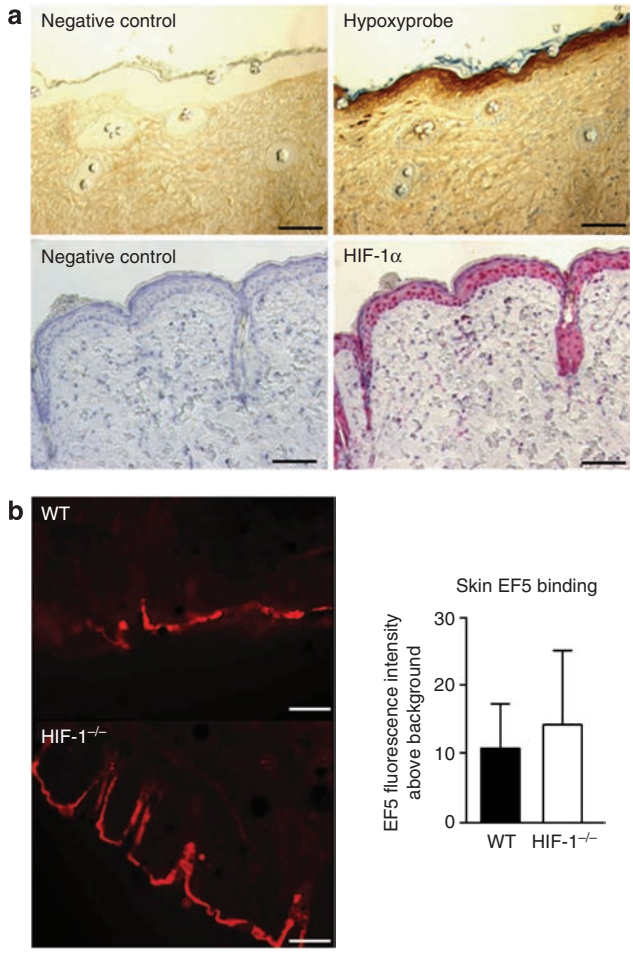

Figure 1. Normal mouse skin is hypoxic and HIF-1α is expressed in the epithelial layer. (**a**) Immunostaining using HypoxyProbe-1 reagent (top) or anti-HIF-1α antibodies (bottom) in WT mouse skin. Bars = 100 μm. Control corresponds to omission of primary antibody. (**b**) Immunofluorescence using the EF5 reagent in WT and HIF-1α$^{-/-}$ (HIF-1α$^{lox/flox}$/K14cre+) mouse skin. Bars = 100 μm.

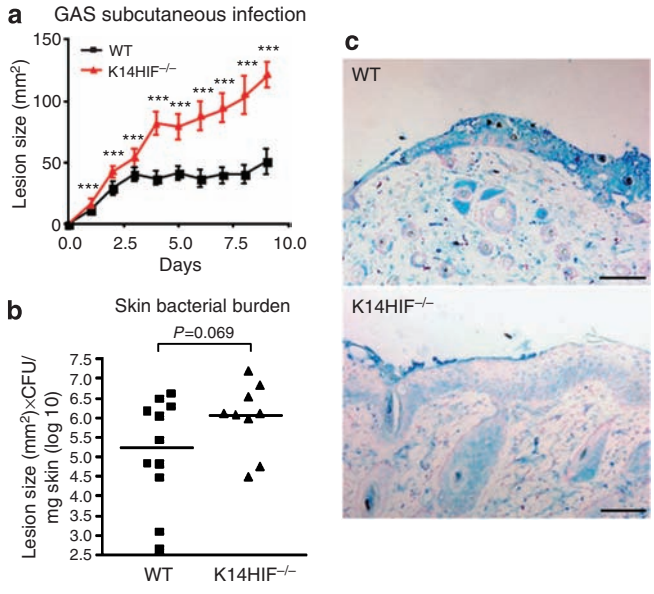

Figure 2. Keratinocyte HIF-1α is important for defense against bacterial infection. (**a**) Measurement of lesion sizes in WT (closed square) and keratinocyte HIF-1α$^{-/-}$ (closed triangle) mice infected subcutaneously with GAS. Ten mice in each group were tested in two paired experiments. Statistical analysis was performed using the unpaired Student's *t*-test; ***$P<0.0001$. (**b**) Bacterial counts in the skin of WT and HIF-1α keratinocyte-null mice infected with GAS. Statistical analysis was performed using the unpaired Student's *t*-test. (**c**) Immunostaining for CRAMP in WT and HIF-1α keratinocyte-null mice skin upon GAS infection. Bars = 100 μm.

(Braff *et al.*, 2005). These gene-encoded ''natural antibiotics'' possess broad-spectrum antimicrobial activity and are produced on epithelial surfaces and within the granules of phagocytic cells. Mice have a single cathelicidin (CRAMP), which closely resembles the single human cathelicidin (LL-37). Importantly, we demonstrated in earlier experiments using the murine model of necrotizing skin infection that endogenous production of CRAMP was essential for mammalian innate immunity to GAS (Nizet *et al.*, 2001). We examined CRAMP production in the biopsies from the necrotic ulcers generated by GAS (Figure 2c). As expected, histopathologic examination revealed a strong CRAMP production by the recruited neutrophils in both the WT and HIF-1α keratinocyte-null mice (in which myeloid HIF is functional). However, CRAMP, strongly expressed in the keratinocytes of WT mice, was absent in HIF-1α keratinocyte-null mice, showing that HIF-1α was important in controlling murine cathelicidin CRAMP production by keratinocytes *in vivo*.

We turned to the human keratinocyte cell line (HaCaT) for further examination of HIF-1α in keratinocyte bactericidal function. HaCaT expression of HIF-1α was targeted for knockdown using a validated small interfering RNA (siRNA) against HIF-1α (1027400; Qiagen, Valencia, CA). HIF-1α mRNA levels were reduced by 85% in HaCaT cells transfected with siRNAHIF-1α (Figure 3a). Correspondingly, HIF-1α protein levels were undetectable following siRNA-HIF-1α transfection (Figure 3b). Exposure of the keratinocyte-anticipated induction was absent in the keratinocytes transfected with siRNAHIF-1α (Figure 3b).

We next examined whether HIF-1α was important in controlling human cathelicidin LL-37 production by the cultured keratinocytes. LL-37 is initially produced as the full-length precursor cathelicidin hCAP-18 (human cathelicidin antimicrobial protein-18) that is later processed by endogenous proteases to the active form (Sorensen *et al.*, 2001). The induction of mRNA transcript for hCAP-18 was diminished in keratinocytes, where HIF-1α expression had been suppressed through siRNA knockdown (Figure 3c). Exposure to the GAS bacteria strongly induced hCAP-18 protein production in HaCaT cells; this response was absent in the keratinocytes transfected with siRNAHIF-1α (Figure 3d).

An independent bactericidal capacity of keratinocytes has been experimentally linked to cathelicidin expression (Braff *et al.*, 2005). To assess the functional consequences of HIF-1α-dependent cathelicidin activation, we performed a bactericidal assay (Braff *et al.*, 2005) with the HaCaT monolayers in the presence or absence of siRNAHIF-1α treatment. In this experiment, 10^5 cfu of GAS were added to each monolayer and aliquots of the supernatant plated over time for to enumerate surviving cfu. Untreated keratinocytes were able to reduce GAS levels in the culture supernatant by 100-fold within 5 hours (Figure 3e). In contrast, in wells exposed to siRNAHIF-1α, levels of viable GAS increased by approximately 100-fold by the 5-hour end point (Figure 3e; $P<0.05$).

In conclusion, our studies establish a role for transcriptional regulator HIF-1α in supporting keratinocyte innate immune function. In the normally hypoxic skin microenvironment, HIF-1α deletion in keratinocytes leads to increased susceptibility to infection with the important skin pathogen GAS. *In vitro* studies in a cultured human keratinocyte cell line demonstrate a role of HIF-1α in supporting inducible cathelidin production and consequent antibacterial activity. Despite the continual array of potential infectious microbes encountered by the mammalian skin, infection remains a rare event. The role of HIF-1α in innate immunity thus appears to extend from supporting phagocyte function in combating established infections, to aiding the skin epithelium in its sentinel barrier function against bacterial invasion.

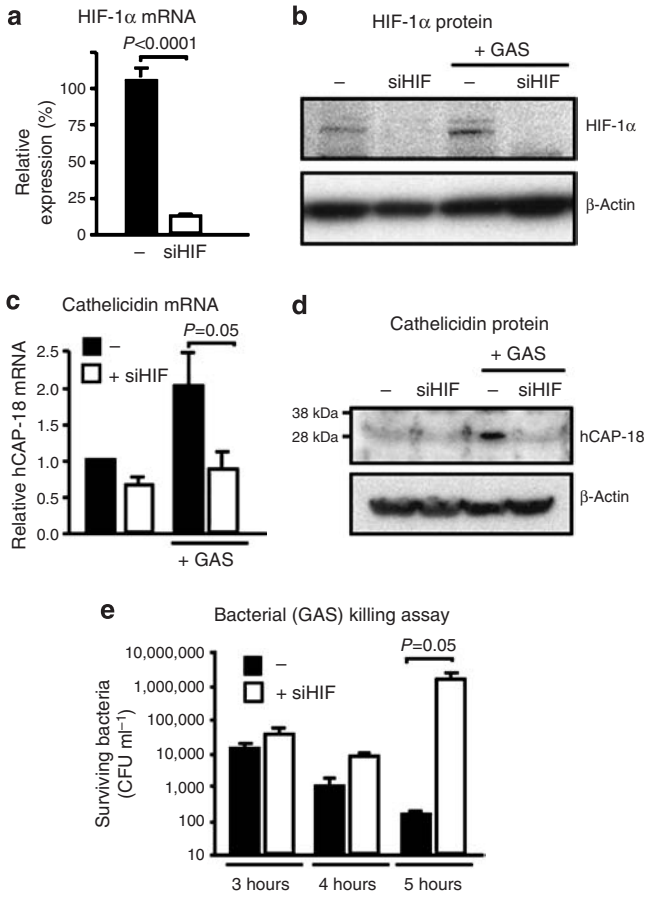

Figure 3. HIF-1α regulates cathelicidin production and bactericidal activity cultured human keratinocytes. (**a**) siRNAHIF-1α treatment of HaCaT cells specifically reduces HIF-1α mRNA expression as seen by real-time reverse transcriptase–PCR. (**b**) Western blot analysis of HaCaT cells ± siRNAHIF-1α ± treatment for 5 hours GAS using anti-HIF-1α antibody (upper panels) or anti-β-actin control antibody (lower panels). (**c**) Real-time reverse transcriptase–PCR for cathelicidin (hCAP18/LL-37) mRNA in HaCaT ± transfection with 5 nM of siRNAHIF-1α ± stimulation for 4 hours with GAS. (**d**) Western blot analysis of HaCaT cells ± transfection with 5 nM of siRNAHIF-α ± stimulation for 4 hours with GAS using LL-37 antibody (upper panels) or anti-β-actin control antibody (lower panels). (**e**) Extracellular killing of GAS (multiplicity of infection, 1 bacterium per cell) by HaCaT transfected ± siRNAHIF-1α. All statistical analyses were performed by unpaired Student's *t*-test. Quantitative assays were performed in triplicate and representative of two or three repeated experiments.

MATERIALS AND METHODS

Animals

Keratinocyte-specific inactivation of HIF-1α (HIF-1α$^{lox/flox}$/K14cre) was achieved by cross-breeding K14-cre transgenic mice (Jonkers *et al.*, 2001) with HIF-1α$^{lox/flox}$ mice. In all experiments, WT littermates from the same breeding pair were used as controls. Mice were handled by approved protocols of the UCSD Animal Care Committee.

Bacterial infection

GAS strain 5448, an M1 serotype isolate from a patient with necrotizing fasciitis (Chatellier *et al.*, 2000), was propagated in Todd–Hewitt media. Our established model of GAS subcutaneous infection was used (Nizet *et al.*, 2001; Datta *et al.*, 2005). After 96 hours, skin lesions were collected and homogenized in (1:1 mg ml^{-1}) phosphate-buffered saline. Serial dilutions of the mixture were plated on Todd–Hewitt broth agar plates for enumeration of cfu.

Immunohistochemistry

Skin tissues were fixed in formalin, sectioned in paraffin, and subjected to microwave antigen retrieval and immunohistochemistry (Nova-Red or alkaline phosphatase blue kits; Vector Laboratories, Burlingame, CA) with primary antibodies against HIF-1α and CRAMP (kind gift from Dr R Abraham and Dr R Gallo, respectively). Development of hypoxic regions within skin lesions was measured using the Hypoxyprobe-1 kit (Millipore, Billerica, MA) as previously described (Peyssonnaux *et al.*, 2005). Nitroimidazole EF5 and the anti-EF5 antibodies were provided generously by Cameron Koch. Mice were injected intraperitoneally with 0.1 ml per 10 g body weight of 10 mM EF5. After 3 hours of binding, mice were killed and tissues were incubated in 30% sucrose overnight. Tissues were frozen in OCT (Tissue-Tek compound (Miles Scientific)) and 14-μm sections were cut on a cryostat. Sections were fixed on the slide in 4% paraformaldehyde for 1 hour. Sections were blocked overnight in 5% mouse serum. Anti-EF5-Cy3 primary antibody or competed antibody (75 μg ml^{-1}) was allowed to bind for 6 hours. EF5 signal was quantified by taking identical 3-second exposures of all primary antibody bound sections as well as their competed antibody-bound counterparts. The competed antibody serves as negative control for autofluorescence and nonspecific binding. The software program ImageJ was used to assign an average fluorescence intensity for each section. Real EF5 fluorescence for each section was calculated by subtracting the competed antibody intensity from the primary antibody intensity.

Real-time reverse transcriptase–PCR and western blot

HaCaT cells, a spontaneously immortalized human keratinocyte line (Boukamp *et al.*, 1988), were propagated in RPM1-1640 media (Sigma, St Louis, MO) supplemented with 10% fetal calf serum. For real-time reverse transcriptase–PCR studies, RNA from HaCaT cells exposed to GAS (multiplicity of infection = 1 bacteria/cell) were extracted and processed as described (Peyssonnaux *et al.*, 2005) (primer sequences available on request). For western blots, HaCaT cells exposed to GAS at a multiplicity of infection of 1 bacterium per cell for 5 hours were harvested and extracted with radioimmunoprecipitation assay buffer and probed using rabbit anti-mouse HIF-1α (Novus Biologicals, Littleton, CO) or anti-LL-37 (Innovagen, Lund, Sweden) antibodies.

siRNA knockdown experiments

Mouse-validated, HIF-1α-specific siRNAs were purchased from Qiagen. Approximately 6×10^4 HaCaT cells were freshly plated in a 24-well format and transfected the following day with 5 nM of HIF-1α siRNA using HiPerFect transfection reagent (Qiagen). A bactericidal assay was performed 48 hours later. Briefly, GAS in logarithmic growth phase were added to cell culture containing antibiotic-free medium at a multiplicity of infection of 1 bacterium per cell. Bacteria were centrifuged onto the cells at 2000 r.p.m. for 5 minutes and incubated at 37 °C. At specified time points, supernatant was collected and dilutions plated on Todd–Hewitt agar for enumeration of cfu.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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