Upregulation of Hypoxia-Inducible Factors in Normal and Psoriatic Skin

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Angiogenesis induced by vascular endothelial growth factor (VEGF) plays an important role in psoriasis. Hypoxic adaptation is conferred through hypoxia-inducible transcription factors (HIFs). VEGF and its receptor Flt-1 are HIF target genes. Growth factors and inflammatory cytokines activate the phosphoinositol-3 kinase pathway, and via activated protein kinase B (phospho-Akt) augment HIF activity. Here, we demonstrate that the major oxygen-dependent HIF isoforms are strongly upregulated in psoriatic skin: HIF-1α mainly in the epidermis, in an expression pattern similar to VEGF mRNA; HIF-2α in both the epidermis and in capillary endothelial cells of the dermis. In contrast, normal human skin shows low expression of HIF-α proteins, with the exception of hair follicles, and glands, which strongly express HIF-1α. In normal human skin, phospho-Akt appeared in the basal epidermal layer, in hair follicles, and in dermal glands. In contrast, in psoriasis, phospho-Akt expression was low in the epidermis, but markedly enhanced in the dermal capillaries and in surrounding interstitial/inflammatory cells. Our data suggest that hypoxia initiates a potentially self-perpetuating cycle involving HIF, VEGF, and Akt activation, which could drive physiologic growth of hair follicles and skin glands. Furthermore, such a cycle may exist in psoriasis in dermal capillaries and contribute to disease progression.

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INTRODUCTION

Psoriasis is a genetically determined chronic inflammatory skin disorder, which is triggered by environmental stimuli. There is increasing evidence that both innate and adaptive immunity are involved, but neither have potential self-antigens been isolated, nor has the specificity of autoreactive skin-infiltrating lymphocytes been defined (reviewed by Bachelez, 2005; Schön and Boehncke, 2005; Gaspari, 2006; Griffiths *et al.*, 2006).

Angiogenesis is a hallmark of psoriasis. Microvessels of the papillary dermis are elongated, tortuous, dilated, and show increased endothelial cell proliferation. Pivotal angiogenic genes like vascular endothelial growth factor (VEGF) and its receptors are upregulated (Detmar *et al.*, 1994). Several lines of evidence suggest that VEGF upregulation is an early and important step in the pathophysiology of psoriasis (reviewed by Detmar, 2004). In mice, chronic transgenic delivery of VEGF to the skin induces inflammation and all the characteristics of psoriasis suggesting a causative role of VEGF in this disease (Xia *et al.*, 2003). Using a potent VEGF antagonist, the VEGF-Trap, reverses this phenotype (Xia *et al.*, 2003). Distinct genetic polymorphisms leading to increased circulating VEGF in healthy humans have been shown to associate with psoriasis (Young *et al.*, 2004). Thus, an ''angiogenic constitution'' might determine psoriasis susceptibility.

Transcriptional activation of VEGF and of its receptor Flt-1 can be achieved by so-called hypoxia-inducible factors (HIFs) (Liu *et al.*, 1995; Gerber *et al.*, 1997). In contrast, the VEGF receptor kinase insert domain receptor (KDR) seems not to be regulated by HIFs (Gerber *et al.*, 1997). HIFs are heterodimers of a constitutive β-subunit and one of at least two different α-subunits (reviewed by Wenger, 2002; Maxwell, 2004; Metzen and Ratcliffe, 2004; Semenza, 2004). Regulation of the HIF system occurs by oxygen-dependent hydrolysis of HIF-α. HIF transcription factors are ubiquitously expressed, instantaneously upregulated upon hypoxia, short-lived upon reoxygenation, and confer cell/tissue protection through many HIF target genes (reviewed by Wenger, 2002; Rosenberger *et al.*, 2005b). The key enzymes of HIF-α proteolysis, so-called HIF prolyl hydroxylases function in the range of physiologic/pathologic oxygen tensions (Epstein *et al.*, 2001), thus fulfilling conditions required for true oxygen sensors. Using high-amplification immunohistochemistry, HIF-1α and -2α have been demonstrated in various rat

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Abbreviations: CO, carbon monoxide; HIF, hypoxia-inducible factor; ISH, in situ *hybridization; KDR, kinase insert domain receptor; SSC, standard saline citrate; VEGF, vascular endothelial growth factor*

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and human tissues (Wiesener *et al.*, 2002, 2003; Rosenberger *et al.*, 2002, 2003, 2005, 2006; Warnecke *et al.*, 2003; Jürgensen *et al.*, 2004; Bernhardt *et al.*, 2006; Goldfarb *et al.*, 2006). Normal tissues were constantly HIF negative, but several hypoxic maneuvers showed that the two isoforms, HIF-1α and -2α, are activated in a cell-type- and stimulus-specific pattern (Rosenberger *et al.*, 2002, 2003, 2005, 2006; Jürgensen *et al.*, 2004; Goldfarb *et al.*, 2006). Comparison with the hypoxia marker pimonidazole (which binds to tissues at oxygen tensions below 10 mm Hg (Franko and Chapmann, 1982) demonstrated that HIF activation occurs in areas which stain positive for pimonidazole, but cells are unable to accumulate HIF in the most severely hypoxic structures (Rosenberger *et al.*, 2005, 2006).

In psoriasis, cell proliferation could increase oxygen consumption, and epidermal thickening could lead to impaired oxygen supply. The aim of this study was to test the hypothesis that intensified hypoxia and consequent HIF activation is involved in psoriatic skin angiogenesis.

RESULTS

Expression of HIF-α in normal human skin

In normal human skin (Figure 1), HIF-1α protein expression was low and focal in the epidermis (Figure 1d). But, hair follicles (Figure 1a), sebaceous glands (Figure 1b), and sweat glands (Figure 1c) abundantly expressed HIF-1α. In addition, HIF-2α protein was rarely detectable, mostly in the dermis (Figure 1g).

Evidence for hypoxia and hypoxic adaptation in rat skin

In concordance with the data in normal human skin, normal rat skin showed occasional epidermal HIF-1α immunostaining and strong HIF-1α staining in hair follicles (Figure 2c). No HIF-2α (Figure 2e) signals were detectable. The hypoxia marker, pimonidazole, confirmed deep hypoxia of hair follicles in normal skin (Figure 2a). To test the potential for HIF activation, we induced systemic hypoxia by exposing rats to 0.1% carbon monoxide (CO, functional anemia) or to 8% ambient oxygen in a hypoxic chamber for 4 hours (Rosenberger *et al.*, 2002). As expected, both maneuvers intensified existing HIF-1α staining (shown for CO in Figure 2c). In addition, some dermal interstitial/endothelial cells became positive for HIF-1α (Figure 2c), as well as for HIF-2α protein (Figure 2f). Pimonidazole adducts became detectable in the basal epidermal layers, as well as in interstitial cells of the dermis (Figure 2b). Interestingly, hypoxia markers were induced to a similar extent by both CO and 8% O_2 (data not shown), suggesting that oxygen diffusion through the stratum corneum was limited.

Upregulation of HIF-α in psoriasis

Psoriatic skin showed marked upregulation of HIF-1α (Figure 1e) and -2α (Figure 1h) protein throughout the epidermis. In addition, in the dermis, HIF-2α (Figure 1i) was strongly activated, and some HIF-1α signals (Figure 1f) appeared as well. High magnification revealed that most HIF-1α and -2α signals located in capillary endothelial cells of dermal papillae (Figure 1f and i, insets).

HIF-1α mRNA was increased in the epidermis of psoriatic skin (Figure 3b).

Transcriptional upregulation of VEGF and its receptor Flt-1 in psoriasis

As detected by *in situ* hybridization (ISH), mRNAs for both VEGF and its receptor Flt-1 were increased in psoriatic skin, as compared with normal human skin. These results confirm previous data (Detmar *et al.*, 1994). In accordance with VEGF and Flt-1 being HIF target genes (Liu *et al.*, 1995; Gerber *et al.*, 1997), VEGF mRNA and HIF-1α colocated in keratinocytes of the epidermis (Figure 3e), whereas Flt-1 mRNA (Figure 3h) and both HIF-α isoforms appeared in capillary endothelial cells of the dermis.

Expression of activated Akt (phospho-Akt) in normal skin and in psoriasis

Phospho-Akt indicates activation of the phosphoinositol-3 kinase pathway, which can occur in response to growth factors like VEGF (Kilic *et al.*, 2006) and tumor necrosis factor-α (Zhou *et al.*, 2003), and was shown to activate HIF *in vivo* (Semenza, 2000). To test whether phosphoinositol-3 kinase activation may account for the HIF-upregulation seen in normal and psoriatic skin, additional phospho-Akt immunostainings were performed. Indeed, in normal skin phospho-Akt was detectable at the sites of maximum HIF-1α immunosignals: in hair follicles (Figure 4c), in sebaceous glands (Figure 4d) and in sweat glands (data not shown). Furthermore, phospho-Akt abundantly appeared in the basal epidermal layer (Figure 4a). Noteworthy, in normal rat or human skin HIF-1α showed no clear predilection for the basal epidermal layer (Figures 1d and 2c). In psoriasis, epidermal phospho-Akt was reduced (Figure 4b), but intense signals appeared in capillary endothelial cells of dermal papillae (Figure 4b and inset therein), coincident with HIF-1α and -2α (compare Figure 1f and i, insets). Additional phospho-Akt located in and surrounding deeper dermal vessels (Figure 5b). Parallel sections revealed strong perivascular phospho-Akt signals in areas of infiltrating CD8 lymphocytes (Figure 5a), suggesting that at least some CD8 lymphocytes activated Akt. By contrast, on parallel sections no clear link between HIF-2α and CD8 signals could be established (Figure 5c).

DISCUSSION

The main findings of this study are 3-fold: first, hypoxia and hypoxia adaptation occur in normal skin, especially in hair follicles and dermal glands (Figure 1a–c); second, HIFs are strongly activated in psoriasis (Figure 1e, f, h, and i), in cell types which express pivotal angiogenic factors (Figure 2e and h); and third, Akt is activated in normal hair follicles and dermal glands (Figure 4c and d), as well as in psoriatic dermal vessels (Figure 4b).

Specificity of hypoxia detection through HIFs

The specificity of HIF-α protein for hypoxia has been challenged, as hypoxia-unrelated factors like inflammatory cytokines, growth factors, and reactive oxygen species have been shown to induce HIF in cell cultures kept under 21%

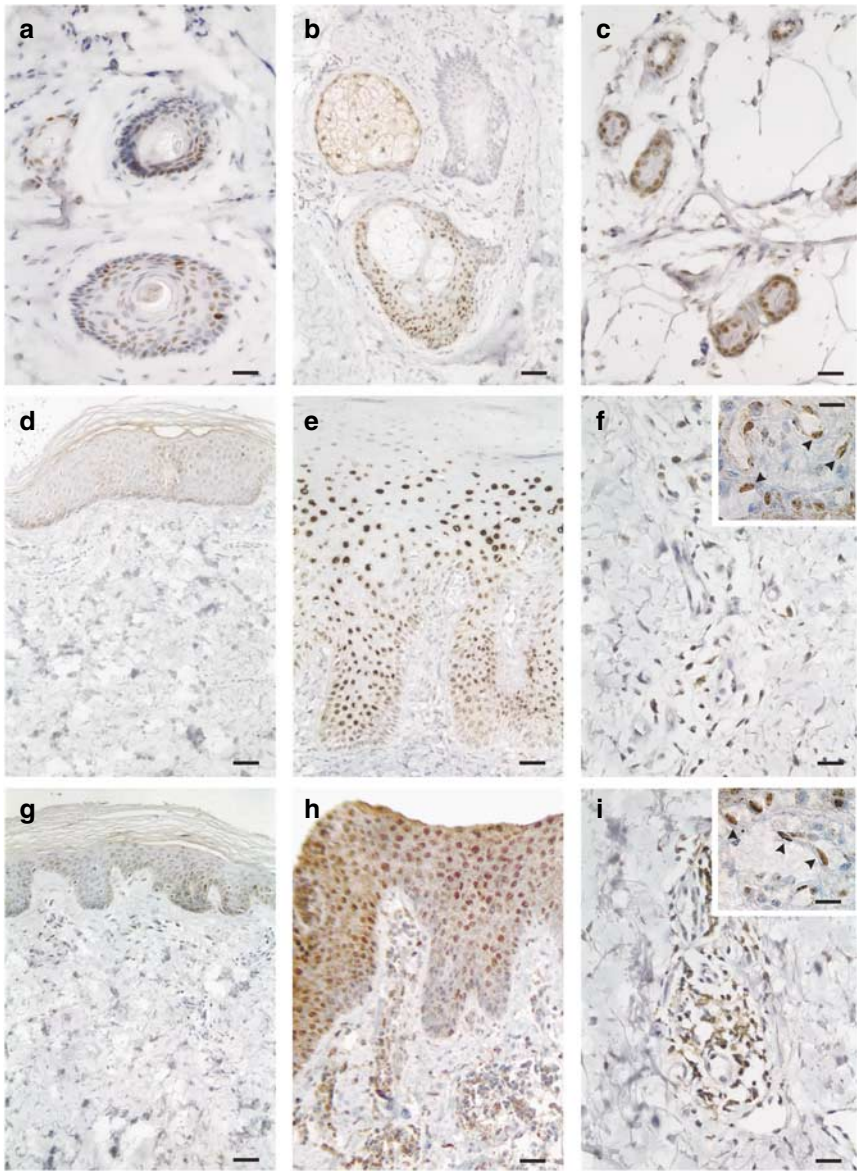

Figure 1. Expression of HIF-1α and -2α protein in normal human skin and in psoriasis. (**a**–**c**) HIF-1α in normal human skin: (**a**) hair follicles, (**b**) sebaceous glands, (**c**) sweat glands, (**d**) epidermis; HIF-2α in normal human skin: (**g**) psoriasis: HIF-1α (**e**) epidermis, (**f**) dermis, inset with higher magnification); HIF-2α (**h**) epidermis, (**i**) dermis, inset with higher magnification). Arrowheads point to endothelial cells. In normal skin, HIF-1α is strongly expressed in (**a**) hair follicles, (**b**) sebaceous glands, and (**c**) sweat glands. (**d**) By contrast, nuclear HIF-1α staining only rarely occurs in normal epidermis. (**g**) Nuclear HIF-2α signals occasionally occur in normal dermis. Psoriasis exhibits (**e**) strong upregulation of HIF-1α in the epidermis and (**f**) to a lesser extent in the dermis. In addition, HIF-2α is upregulated (**h**) in the epidermis and (**h** and **i**) in the dermis. Bars = (**a**–**e**, **g**, **h**) 600 μm; (**f** and **i**) 150 μm; insets in (**f** and **i**) 50 μm.

oxygen (Haddad and Harb, 2005; Hellwig-Burgel *et al.*, 2005; Maranchie and Zhan, 2005). However, the fact that in normal rat skin HIF-1α protein colocates with pimonidazole (Figure 2) strongly suggests hypoxia-dependent HIF stimulation. Unfortunately, we were unable to use pimonidazole in our human skin biopsies, and therefore cannot rule out that factors other than hypoxia had (at least partly) been responsible for HIF activation in psoriasis.

Hypoxia and hypoxia adaptation occur in normal skin

Several lines of evidence suggest that oxygen tensions are low in normal skin. Distler *et al.* (2004) and Bedogni *et al.* (2005) demonstrated nuclear HIF-1α in the epidermis of humans and mice. Cobb *et al.* (1990) found accumulation of tritium-labeled pimonidazole in sebaceous glands. Bedogni *et al.* (2005) also showed accumulation of EF5 (a bioreductive agent like pimonidazole) in the epidermis and hair follicles of normal mice. This study confirms the presence of regional hypoxia and HIF activation in normal skin (Figure 2a and c) and extends previous findings by showing a strong activation of HIF-1α in hair follicles and dermal glands (Figure 1a–c). Noteworthy, in rat skin HIFα immunosignals were enhanced to a similar extent by both CO admixture to room air and global hypoxia (8% ambient oxygen), suggesting that vascular

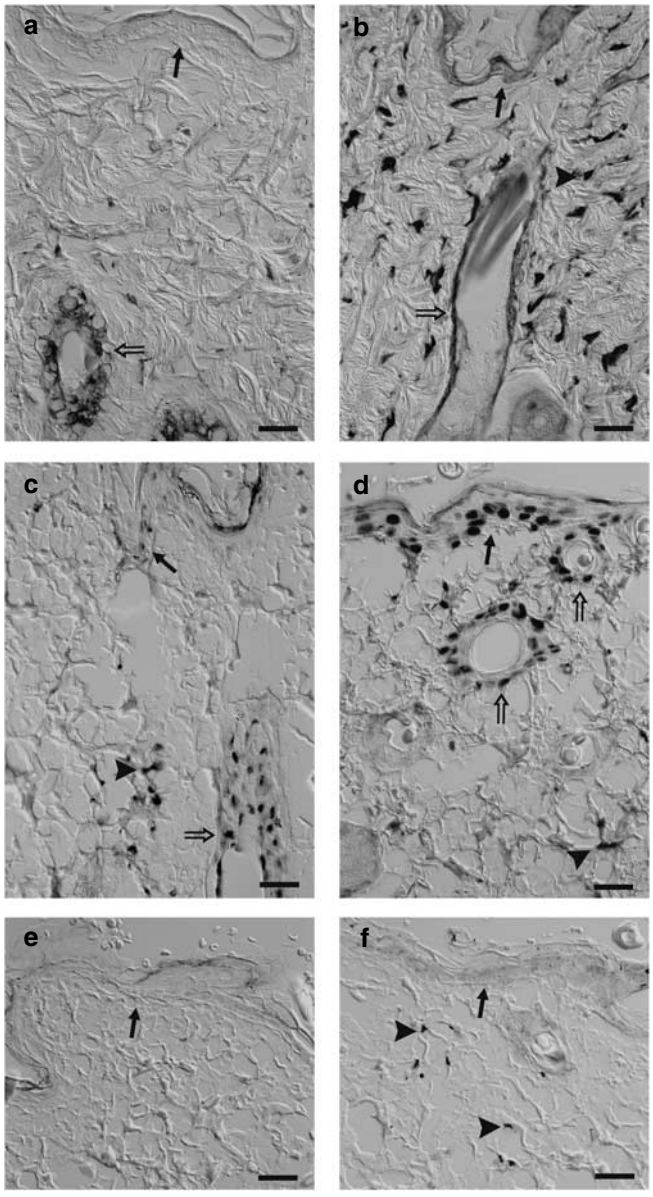

Figure 2. Immunohistochemical evidence for hypoxia in rat skin. Left panels: controls; right panels: hypoxia (0.1% CO); (**a** and **b**) pimonidazole; (**c** and **d**) HIF-1α; (**e** and **f**) HIF-2α. Arrows point to the epidermis; arrowheads = interstitial/endothelial cells; open arrows = hair follicles. (**a**) In normal rat skin, pimonidazole adducts prove deep hypoxia in hair follicles. (**c**) HIF-1α protein appears in hair follicles, and occasionally in the epidermis, (**e**) but staining for HIF-2α is negative. After 4 hours of functional anemia (0.1% CO in hypoxic chamber) hypoxia is intensified. (**b**) Pimonidazole adducts appear *de novo* in interstitial cells of the dermis, and in the epidermal layer. (**d**) HIF-1α signals are more abundant in the epidermis, and some interstitial signals appear in the dermis, as well. (**f**) In addition, some interstitial/endothelial cells of the dermis stain positive for HIF-2α. Bar = 240 μm.

oxygen supply was critical to the skin. Moreover, to our knowledge this is the first report of Akt activation in normal hair follicles and dermal glands (Figure 4c and d). As reviewed by Detmar (2000), the vasculature of adult skin remains normally quiescent due to the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. But, skin retains the capacity for brisk initiation of angiogenesis. Cyclic vascular expansion due to keratinocyte-derived VEGF occurs in the growth phase of hair follicles. VEGF can augment HIFα mRNA translation into protein via phosphoinositol-3 kinase and Akt (Semenza, 2000; Kilic *et al.*, 2006). To our knowledge, there is no evidence for Akt activation by hypoxia *per se*. Possibly, within the physiologic cycle of hair follicles and dermal glands hypoxia activates HIF, HIF activates VEGF, VEGF activates Akt, and Akt augments HIF-response, thus forming a positive feedback loop.

HIF and Akt are activated in psoriasis

This is the first report of HIF activation in psoriasis. HIFs regulate both VEGF (Liu *et al.*, 1995) and its receptor Flt-1 (Gerber *et al.*, 1997), which play a pivotal role in psoriatic angiogenesis (reviewed by Detmar, 2004). We show that HIFα colocates with VEGF in the epidermis. In addition, we demonstrate that in psoriasis HIFα, Flt-1, and activated Akt all occur in dermal capillaries (insets in Figures 1f, i, and 4b).

HIF expression is pronounced in perivascular areas where one would expect the least hypoxia. However, as oxygen level is a balance between supply and consumption, in these perivascular areas oxygen consumption (e.g., by inflammatory cells, stromal cells, endothelial cells, etc.) might exceed oxygen supply, thus leading to relative hypoxia.

Several lines of evidence suggest a crucial role for augmented VEGF response and the so-called ''angiogenesis predisposition'' in the pathophysiology of psoriasis (reviewed by Detmar, 2004). However, it remains unclear, whether such predisposition also includes alterations upstream of the VEGF gene, such as the HIF system.

Noteworthy, mice with transgenic cutaneous VEGF upregulation are the only animal models capable of reproducing the complete psoriatic phenotype (Xia *et al.*, 2003). In these mice, psoriatic plaques evolve spontaneously after a period of approximately 6 months, which is prevented by VEGF inhibition (Xia *et al.*, 2003). By contrast, short-time delivery of VEGF to the skin does not produce psoriatic lesions (Sundberg *et al.*, 2001). Such data suggest that long-term cutaneous VEGF activation is necessary and sufficient to induce psoriasis. By contrast, inflammatory cytokines (like tumor necrosis factor-α) or keratinocyte growth factors (like transforming growth factor-α) alone do not induce the complete psoriatic phenotype, as demonstrated by various transgenic mouse models (reviewed by Xia *et al.*, 2003).

These data offer a first hint that HIFs and Akt could be important players in psoriatic angiogenesis, which theoretically would fit into the existing plot of inflammation and epithelial proliferation.

Additionally, T lymphocytes play an important role in the pathophysiology of psoriasis. Interestingly, T lymphocytes from psoriasis patients induce typical psoriatic plaques in xenografts of uninvolved psoriatic skin, but not in xenografts of normal skin, as shown in the severe combined immune deficiency mouse model (reviewed by Schön and Boehncke, 2005). Theoretically, HIF activation in T cells may contribute

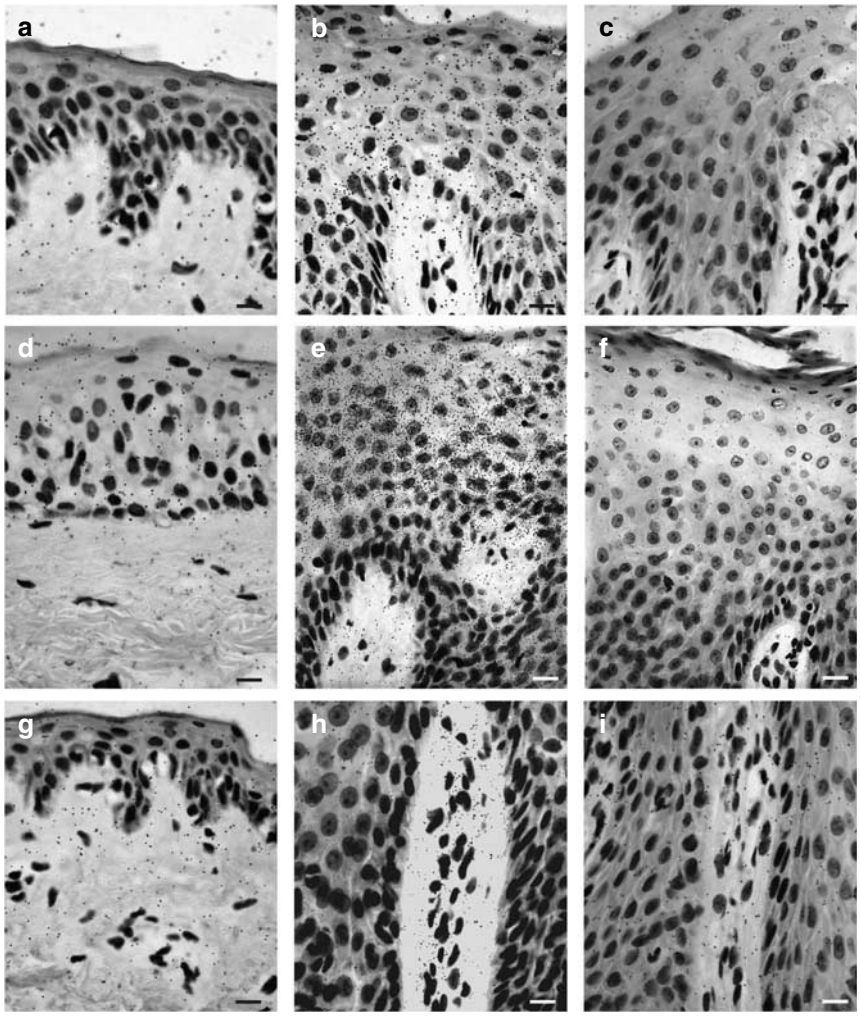

Figure 3. ISH for HIF-1α, VEGF, and its receptor Flt-1 in normal and psoriatic human skin. Left panels: normal skin, antisense probes; mid panels: psoriasis, antisense probes; right panels: psoriasis, sense probes. (**a**–**c**) HIF-1α; (**d**–**f**) VEGF; (**g**–**i**) Flt-1. Staining with (**a**, **d**, **g**) antisense probes in normal skin is not different from staining with sense probes (exemplified for psoriasis in **c**, **f**, **i**), which is consistent with background activity. On the contrary, all three antisense probes show more abundant signals in psoriasis: HIF-1α and VEGF in the epidermis, and Flt-1 in endothelial cells of dermal vessels. Bar = 150 μm.

to psoriatic pathophysiology, given that HIF is involved in T-cell survival and function (Kojima *et al.* 2003; Makino *et al.*, 2003; Nakamura *et al.* 2005).

Nevertheless, our data suggest that, at least in established psoriatic plaques, most CD8 lymphocytes are HIF-negative, as perivascular CD8 lymphocytes expressed phospho-Akt but not HIFα. However, as immunostaining for HIF and CD8 were incompatible in terms of antigen retrieval, we were only able make a rather rough estimate based on parallel sections. Moreover, we cannot exclude HIF activation in T cells at earlier stages of psoriasis.

Conclusion

Physiologic regional hypoxia occurs in dermal glands and hair follicles, possibly initiating a cycle involving HIF, VEGF, and Akt activation, which has the potential for self-perpetuation. Most likely, skin hypoxia is further intensified in psoriasis, where keratinocyte and endothelial HIF could promote angiogenesis by differentially upregulating VEGF and its receptor Flt-1, respectively. Moreover, in dermal capillaries, phospho-Akt likely provides a positive feedback to the HIF/VEGF system.

MATERIALS AND METHODS

The study was conducted according to the Declaration of Helsinki Principles.

Human tissue

Biopsies of involved skin from 10 patients with psoriasis and of unremarkable skin from five control patients were obtained after written informed consent. Institutional approval was not necessary for the human studies, as all biopsies were diagnostic. Biopsies were fixed for 1 hour in freshly depolimerized 3% paraformaldehyde (in phosphate-buffered saline, pH 7.4) at 4°C, after which they were transferred into ice-cooled phosphate-buffered saline, and embedded into paraffin within 2–6 hours. Additional 10 archival biopsies from psoriatic patients and three frozen biopsies from psoriatic skin were included.

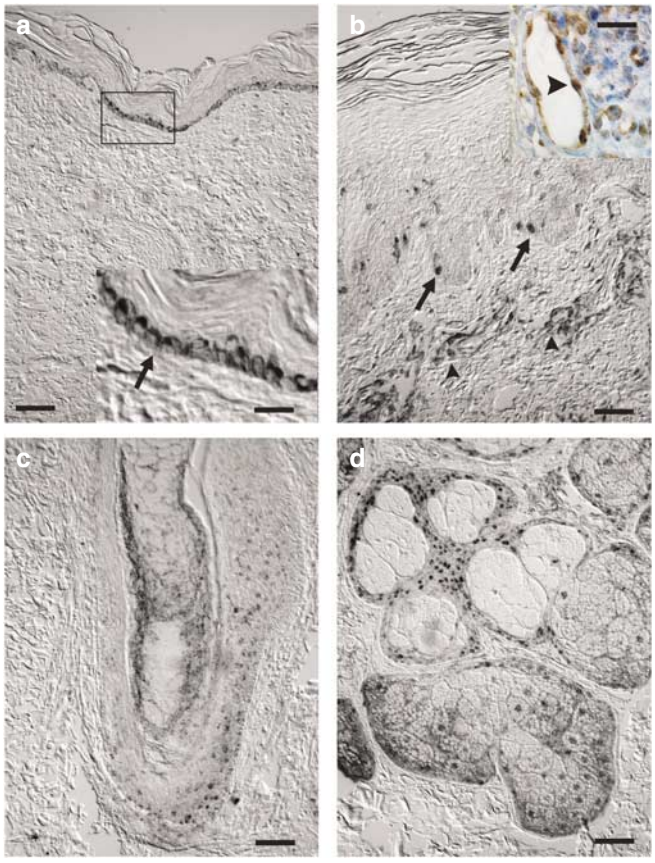

Figure 4. Expression of activated Akt (phospho-Akt) in normal and psoriatic human skin. Control skin: (**a**, **c**) hair follicles, (**d**) sebaceous glands; (**b**) psoriasis. Arrows point to the epithelium; arrowheads = interstitial/endothelial cells. Inset in panel a is a high-power magnification of the outlined area. In normal skin, phospho-Akt is detectable in the basal layer of (**a**) the epidermis, (**c**) in hair follicles, and (**d**) in sebaceous glands. In psoriasis, epidermal phospho-Akt is rarer, but intense dermal signals appear (**b** and high power inset therein). Bars = (**a**–**d**) 600 μm; (inset in **a**) 60 μm; (inset in **b**) 50 μm.

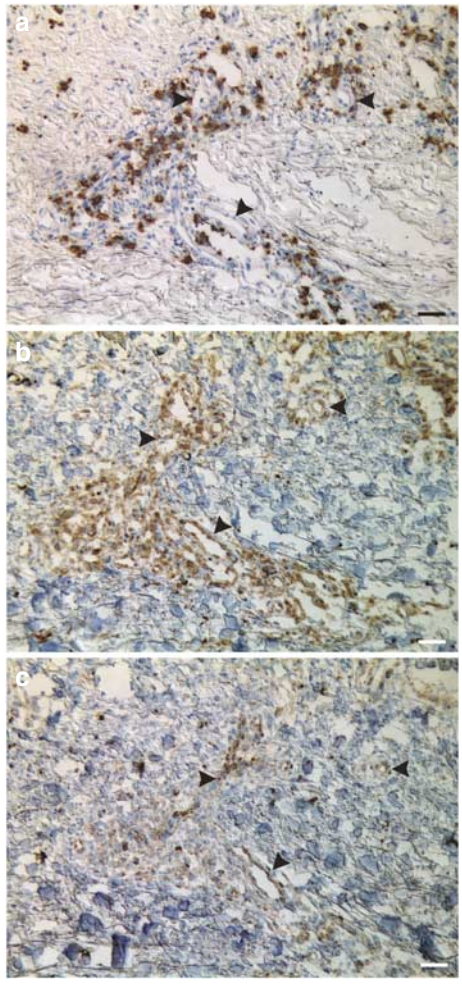

Figure 5. Perivascular CD8 lymphocytes in psoriasis. Six micrometers parallel cryostate sections. (**a**) CD8, (**b**) phospho-Akt, (**c**) HIF-2α. Signals appear brown; blue counter staining with hematoxylin. Arrowheads indicate corresponding capillaries. Owing to antigen retrieval (pressure cooker), tissue is altered in (**b**) and (**c**). (**a**) CD8 lymphocytes appear in clusters surrounding dermal vessels. (**b**) Phospho-Akt signals locate in endothelial cells and in the surroundings of vessels, in areas of CD8 positivity, suggesting that at least some CD8 lymphocytes had activated Akt. (**c**) By contrast, nuclear HIF-2α signals were rare in this area, and mostly located in endothelial cells. Our data suggest that CD8 lymphocytes did not activate HIF. Bar = 270 μm.

Animal studies

The study was approved by the Institutional Review Board for the care of animal subjects, and was performed in accordance with National Institute of Health guidelines. Six male Sprague–Dawley rats (Winkelmann, Borchem, Germany) were used at weights of 200–280 g. For the induction of hypoxia (functional anemia), animals were placed in an air-tight Plexiglas cabinet, and exposed for 4 hours to normal air with 0.1% CO, or to a mixture of 8% O_2 and 92% N_2 (Rosenberger *et al.*, 2002). For the detection of pimonidazole adducts as markers of tissue hypoxia, pimonidazole (Hypoxyprobe, Natural Pharmacia International, Belmont, MA) was delivered intravenously at 60 mg/kg at 1 hour before euthanasia (Rosenberger *et al.*, 2003). In the case of hypoxic induction, animals were briefly taken out of the hypoxic chamber in order to receive their pimonidazole injection. Tissue were fixed and embedded into paraffin, as detailed above.

Immunohistochemistry

Staining with mouse-anti-human HIF-1α (which cross-reacts with rat HIF-1α; α67, Novus Biologicals, Littleton, CO; 1:10,000), rabbit-anti-rat HIF-2α (PM9, gift from Patrick Maxwell, Oxford, UK; 1:10,000), rabbit-anti-human HIF-2α (190b, gift from Patrick Maxwell, Oxford, UK; 1:1,000), and mouse-anti-pimonidazole (Hypoxyprobe, Natural Pharmacia International, Belmont, MA; 1:1,000) was performed as described previously (Rosenberger *et al.* 2002, 2003). Staining for phospho-Akt (Ser-473) was performed with a rabbit polyclonal antibody (1:1,000) from Cell Signaling Technology (Beverly, MA; cat. no. 3787, immunohistochemistry specific) after antigen retrieval (25 minutes boiling in 0.01 M citrate buffer, pH 6.0). Staining for CD8 was performed on formalin-fixed cryostate sections using mouse-anti-human CD8 (clone CLB-T8/4, 4H8, PeliCluster, Amsterdam, The Netherlands; 1:1,000).

ISH

ISH was performed on 5 μm paraffin sections. Details of ISH have been published previously (Brown *et al.*, 1992). Briefly, slides were

passed through xylene, graded alcohols, 0.2 M HCl; Tris/EDTA with 3 μg/ml proteinase K; 0.2% glycine; 4% paraformaldehyde in phosphate-buffered saline, pH 7.4; 0.1 M triethanolamine containing 1/200 (vol/vol) acetic anhydride; and 2 × standard saline citrate (SSC). Slides were hybridized overnight at 50°C with ^{35}S-labeled riboprobes in the following mixture: 0.3 M NaCl, 0.01 M Tris, pH 7.6, 5 mM EDTA, 50% formamide, 10% dextran sulfate, 0.1 mg/ml yeast tRNA, and 0.01 M dithiothreitol. Post-hybridization washes included 2 × SSC/50% formamide/10 mM dithiothreitol at 50°C; 4 × SSC/10 mM Tris/1 mM EDTA with 20 μg/ml ribonuclease A at 37°C; and 2 × SSC/50% formamide/10 mM dithiothreitol at 65°C and 2 × SSC. Slides were then dehydrated through graded alcohols containing 0.3 M ammonium acetate, dried, coated with Kodak NTB 2 emulsion, and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer and the slides were counterstained with hematoxylin. Antisense 204 bp single-stranded ^{35}S-labeled VEGF-A RNA probe and its sense have been described previously (Brown *et al.*, 1992). The antisense probe hybridizes specifically with a region of VPF/VEGF mRNA common to all known VPF/VEGF splicing variants. ^{35}S-labeled single-stranded 225 bp antisense probe targeted to the kinase insert region and sense RNA probes for the VEGF-A receptor VEGFR-1 (vascular endothelial growth factor receptor-1, Flt-1) have been described previously (Brown *et al.*, 1993). Probes for HIF-1α (246 bp) were obtained from 737–983 bp of the cDNA, with both sense and antisense orientation confirmed by sequencing.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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