学位論文

Psoriasis-like skin inflammation is reduced in transgenic mice overexpressing inhibitory PAS domain protein

（Inhibitory PAS domain protein 過剰発現マウスでは乾癬様皮膚炎が減弱する）

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Research Notes

Psoriasis-like skin inflammation is reduced in transgenic mice overexpressing inhibitory PAS domain protein

Running title: Psoriasis in mice overexpressing IPAS

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Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that functions as a major regulator of gene expression in hypoxia. HIF-1 is a heterodimeric complex comprising two subunits, HIF-1α and HIF-1β. Under hypoxic conditions, HIF-1α is stabilized via inhibition of proteasomal degradation; it subsequently dimerizes with HIF-1β. This complex interacts with the hypoxia-responsive elements (HRE) in the promoter region of the target genes such as vascular endothelial growth factor (VEGF) to activate transcription [1]. The inhibitory PAS domain protein (IPAS), an alternatively spliced variant of HIF-3α in mice, acts as a dominant negative inhibitor of HIF-1α [2, 3]. IPAS forms an abortive complex with HIF-1α with regard to its ability to recognize HRE and thus impairs HIF-1α target gene expression in response to hypoxia. IPAS is abundantly found in the corneal epithelium of the eye, which correlates with low level of hypoxia-inducible VEGF expression and maintenance of the avascular phenotype. Moreover, IPAS is expressed in primary mouse epidermal keratinocytes [4]; however, the role of IPAS/HIF-3α in the keratinocytes remains unknown. HIF-1α plays an important role in several skin disorders such as cutaneous angiogenesis and skin tumorigenesis [1]. Previous studies reported that HIF-1α is abundantly expressed in human psoriatic epidermis and promotes angiogenesis by inducing VEGF [5]. Here, we investigated the role of IPAS/HIF-3α in the development of human psoriasis and in a murine model of

psoriasis.

To investigate the role of IPAS/HIF-3α in psoriasis development, we initially examined the expression of HIF-1α and HIF-3α in human psoriasis. In accordance with the previous report [5], HIF-1α was found to be localized in the nucleus of normal human epidermis in the immunohistochemistry analysis. Furthermore, its expression was increased in the psoriatic epidermis (Fig. 1a). In contrast, western blotting revealed that HIF-3α expression was reduced in human psoriatic skin when compared with normal skin (Fig. 1b). In accordance with this, immunohistochemistry also showed that, while HIF-3α protein was located in the nucleus of normal human epidermis, its expression was decreased in the psoriatic epidermis (Fig. 1c). Furthermore, we examined HIF-1α and IPAS/HIF-3α expression in imiquimod (IMQ)-induced murine psoriasiform dermatitis. In immunohistochemistry analysis, HIF-1α expression was increased in the nucleus of keratinocytes, suggesting its activation in IMQ-treated epidermis (Fig. 1d). Quantitative RT-PCR analysis confirmed that the expression of VEGF, a gene upregulated by HIF-1α, was elevated in IMQ-treated skin. In contrast, IPAS/HIF-3α expression was reduced in the IMQ-treated skin when compared with untreated skin (Fig. 1e). These results indicate that IPAS/HIF-3α expression is downregulated in murine psoriasiform dermatitis as well

as in human psoriasis.

To further investigate the role of IPAS/HIF-3α, we developed a novel transgenic (Tg) mouse model overexpressing IPAS and controlled by the cytomegalovirus promoter (Supporting information). Previous reports have revealed that HIF-1α activation in keratinocytes crucially affects skin inflammation [6]. Therefore, we hypothesized that IPAS/HIF-3α represses psoriasiform dermatitis by inhibiting HIF-1α activation. To confirm this hypothesis, we examined the development of IMQ-induced psoriasiform dermatitis in IPAS-Tg mice. Although IPAS-Tg mice developed skin lesions similar to those in wild type mice, acanthosis was significantly lower in IPAS-Tg mice (Fig. 1f, g). Quantitative RT-PCR revealed that the IMQ-induced expression of VEGF was markedly reduced in the skin of IPAS-Tg mice (Fig. 1h), suggesting that the overexpression of IPAS represses the HIF-1α-VEGF pathway. Furthermore, the Ki67-positive keratinocyte count was decreased in the IMQ-treated IPAS-Tg mice skin (Fig. 1i, j), indicating that IPAS suppresses keratinocyte cell proliferation in psoriasiform dermatitis. These results indicate that IPAS/HIF-3α suppresses skin inflammation by inhibiting HIF-1α activity in psoriasiform dermatitis.

In this study, we demonstrated that the expression of IPAS/HIF-3α, a dominant negative regulator of HIF-1α, is downregulated in psoriasiform epidermis. Furthermore, we presented that IPAS overexpression represses skin inflammation potentially by inhibiting the HIF-1α-VEGF pathway in the IMQ-induced psoriasiform dermatitis. Collectively, our data suggest that downregulation of IPAS/HIF-3α plays an important role in the pathogenesis of psoriasiform dermatitis by promoting HIF-1α-mediated VEGF expression. Angiogenesis induced by VEGF is essential in psoriasis. Indeed, transgenic mice overexpressing VEGF in keratinocytes develop a phenotype similar to psoriasis suggesting the causative role of VEGF in this disease [7]. Furthermore, the HIF-1α-VEGF pathway also plays an important role in skin pathophysiology, such as wound healing and skin tumorigenesis. Therefore, understanding the regulatory mechanisms of HIF-1α activation in the skin is important. To the best of our knowledge, this is the first study to emphasize the importance of IPAS/HIF-3α in the regulation of HIF-1α activity in the skin. Further studies are required to clarify the expression and function of IPAS/HIF-3α in various human skin diseases including psoriasis.

Conflict of interest

The authors declare no conflict of interest.

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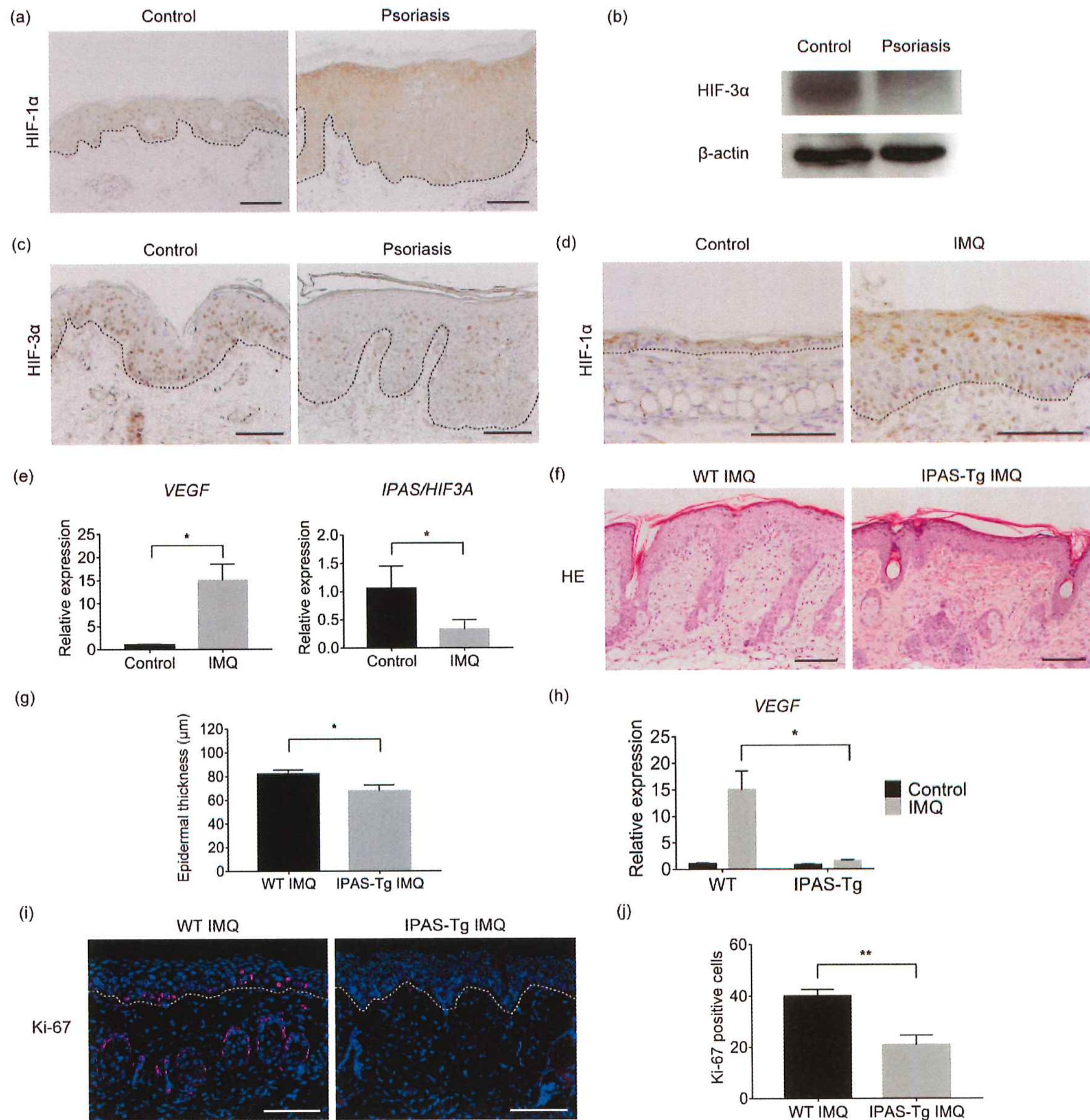
Figure Legends

Figure 1

(a) Immunohistochemistry analysis of HIF-1α (rabbit anti-HIF-1α antibody) in normal skin of the healthy control and the lesional skin of psoriasis. (b) The expression of HIF-3α was evaluated through western blotting (rabbit anti-HIF-3α antibody) in normal skin of the healthy control and the lesional skin of psoriasis. (c) Immunohistochemistry analysis of HIF-3α in normal skin of the healthy control and the lesional skin of psoriasis. (d) The back skin of wild type (WT) mice were treated with imiquimod (IMQ) cream for 6 consecutive days. HIF-1α expression on the back skin was analyzed via immunohistochemistry on day 0 and day 6. Scale bar: 100 μm. (e) The expression of vascular endothelial growth factor (VEGF) and inhibitory PAS domain protein (IPAS) was analyzed via quantitative RT-PCR in IMQ-treated back skin on day 0 (Control) and day 6 (IMQ). Data represent means ± SEM from four mice per group. $*p < 0.05$. (f) WT and IPAS-transgenic (Tg) mice were treated with IMQ cream on the back skin for 6 consecutive days. Hematoxylin–eosin staining of WT and IPAS-Tg back skin sections on day 6. Scale bar: 100 μm. (g) The thickness of epidermis was measured in hematoxylin and eosin-stained skin sections of IMQ-treated WT and IPAS-Tg mice. (h) The expression of VEGF was analyzed via quantitative RT-PCR in IMQ-treated back skin on day 0

(Control) and day 6 (IMQ). (i, j) The number of epidermal Ki-67-positive cells in representative high power fields (HPF) of IMQ-treated WT and IPAS-Tg back skin sections was enumerated on day 6. Data represent means ± SEM from four mice per group. $^{**}p < 0.01$. The dashed lines represent the dermal–epidermal junction.

Figure 1



Materials and Methods

IPAS transgenic mouse

To generate a transgenic mouse, full-length inhibitory PAS domain protein (IPAS) cDNA was inserted downstream of the FLAG-tag coding sequence in the pFLAG-CMV2 vector [S1], and the pFLAG-CMV2-IPAS plasmids were microinjected into mouse eggs in accordance with the standard microinjection procedures for transgenic mouse production (Oriental Yeast, Tokyo, Japan). Groups of founders from the F0 generation were genotyped via PCR using the primers Fw (5′-ACCATGGACTACAAAGACGA-3′) and Rv (5′-GAGCTCCAGAGAGAAATTGG-3′). The transgenic mice were backcrossed for more than 10 generations to C57BL/6J mice (Japan SLC, Hamamatsu, Japan), and the FLAG-IPAS-expressing offspring mice were used in this study. All animal experiments were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (Protocol No. 17168) and the Safety Committee for Recombinant DNA Experiments (Protocol No. 15-14) of the Asahikawa Medical University.

Imiquimod-induced psoriasis model

Age- and sex-matched mice (at six weeks of age) were used for these experiments. For inducing psoriasiform dermatitis, the ears and shaved backs of mice were treated up to 6 consecutive days with commercially available 5% imiquimod cream (Beselna Cream; Mochida Pharmaceutical, Tokyo, Japan), as described previously [S2].

Patient samples

A total of three patients with psoriasis vulgaris, at Asahikawa Medical University Hospital, participated in this study. The samples were collected with an approval of the local ethical committee and the institutional review board of Asahikawa Medical University, and all patients gave their written informed consent.

Histology and Immunohistochemistry

Mouse skin was fixed in 20% formalin and embedded in paraffin. Paraffin sections were processed for standard hematoxylin and eosin staining for histological analysis. For immunofluorescence staining, mouse skin was embedded in an optimal cutting temperature compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and stored at −80 °C until further use. Sections were fixed in acetone for 10

min at −20 °C and were incubated with primary antibody overnight in a humidified chamber at 4 °C. The following primary antibodies were used: rabbit anti-HIF-1α (1:100; Novus Biologicals, Littleton, CO), rabbit anti-HIF-3α (1:100; Abcam, Cambridge, MA) and rat anti-Ki-67 (1:100; eBioscience, San Diego, CA). Tissues were subsequently labeled with the Alexa Fluor 594-coupled secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h at room temperature and the nuclei were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA). Images were obtained using a confocal laser microscope (Fluoview FV1000D; Olympus, Tokyo, Japan) and were analyzed via ImageJ software (NIH Image, Bethesda, MD).

Western blotting

Total protein was isolated from the mouse skin and lysed in RIPA buffer comprising a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL). Proteins (50 μg per lane) were separated via SDS-PAGE and were transferred to Hybond-PVDF membranes (Amersham Bioscience, Piscataway, NJ). The blotted membranes were blocked with Tris-buffered saline containing 0.1% Tween-20/10% skimmed milk and were then incubated with primary antibodies of HIF-3α (1:1000;

Abcam, Cambridge, MA) overnight at 4 °C. Equal protein loading was demonstrated by probing the membranes with a rabbit anti β-actin antibody (1:2000; Novus Biologicals, Littleton, CO). Proteins were visualized with anti-rabbit IgG horseradish-peroxidase-linked antibodies (Amersham Bioscience) for 1 h, followed by chemiluminescence detection (ECL Plus, Amersham Bioscience).

Real-time quantitative PCR

Total RNA was isolated from the mouse skin according to the RNeasy Mini Kit (Qiagen, Hilden, Germany) protocol and was reverse transcribed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using LightCycler 480 System (Roche Diagnostics, Basel, Switzerland) and the following primer sets: Vegfa: Fw (5′-TAACTCAAGCTGCCTCGCC-3′) and Rv (5′-GAGCCTTGTTCACAGCGGA-3′); Ipas: Fw (5′-TGGTACCAGGAGTGGGGTTA-3′) and Rv (5′-AGGTGGAAAAAGGGGGAGAG-3′); Actb: Fw (5′-ACCAGAGGCATACAGGGACA-3′) and Rv (5′-CTAAGGCCAACCGTGAAAAG-3′). Data were normalized to the Actb housekeeping gene.

Statistical analyses

Statistical significance of the data obtained was assessed via two-tailed Student t test or one-way ANOVA with Tukey's or Bonferroni's multicomparison test, using GraphPad Prism 7.00 software (GraphPad Software, San Diego, CA).

References

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Figure Legends

Figure S1

(a) Schematic structure of the transgene. The mouse full-length inhibitory PAS domain protein (IPAS) cDNA was fused at its 5′ end with the FLAG epitope and was cloned downstream of the regulatory sequences of the cytomegalovirus promoter. (b) Transgenic mice were discriminated from wild type (WT) animals by genomic PCR using primers specific for the SV40 intron and the FLAG epitope. Interleukin-2 amplification was used as an internal control. (c) Transgenic IPAS expression was confirmed via western blot analysis using anti-Flag antibodies on the skin protein extracts. (d) Hematoxylin–eosin staining of WT and IPAS-Tg back skin sections. Scale bar: 100 μm.

Figure S2

(a, b) The expression of IPAS/HIF-3α and HIF-1α was analyzed via quantitative RT-PCR in IMQ-treated WT and IPAS-Tg mice back skin on day 0 (Control) and day 6 (IMQ). (c) HIF-1α expression was analyzed using immunohistochemistry in IMQ-treated WT and IPAS-Tg mice back skin on day 0 (Control) and day 6 (IMQ). Scale bar: 100 μm. The dashed lines represent the dermal–epidermal junction.

Figure S1

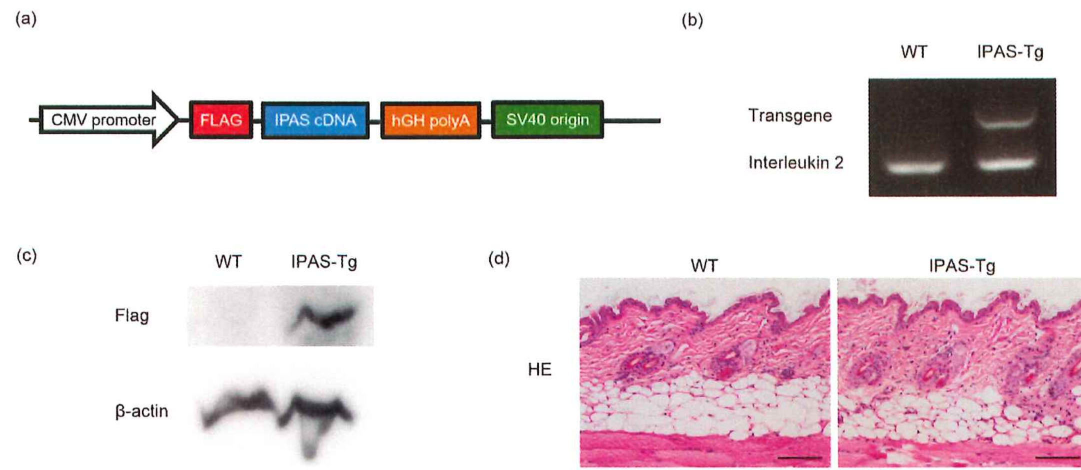

Figure S2