Experimental dermatology • **Original article**

CED
Clinical and Experimental Dermatology

Understanding the role of haptoglobin in psoriasis: effects of ultraviolet B

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doi:10.1111/ced.12667

Summary

Background. Haptoglobin (Hp) is one of the acute phase proteins, whose main function is to bind free haemoglobin (Hb) and transport it to the liver for degradation and iron recycling. In addition to its role as an Hb scavenger, Hp has been shown to behave as an anti-inflammatory, antioxidant and angiogenic factor. We previously investigated the role of Hp in the pathogenesis of psoriasis, and found that it displays some structural modifications that might be associated with protein function in the disease. Phototherapy is an efficacious treatment for psoriasis, although the biological mechanisms by which phototherapy improves psoriasis are still unclear.

Aim. To investigate the effects of ultraviolet (UV)B on Hp to clarify the role of Hp in psoriasis.

Methods. Expression of the genes encoding Hp, interleukin (IL)-6 and IL-10 was assessed in UVB-irradiated and unirradiated HaCaT cells. The biological significance of Hp modulation of UVB treatment was confirmed by ELISA and Western blotting. The Hp gene and protein expression in the skin of patients with psoriasis was also investigated.

Results. *In vitro* results showed that UVB modulated IL-6 and IL-10 gene expression and Hp gene and protein expression in HaCaT cells. The *in vivo* data also showed that Hp levels were increased in the skin of patients with psoriasis compared with healthy controls.

Conclusions. UVB irradiation was able to modulate Hp production in immortalized keratinocytes. The higher levels of Hp *in vivo* in both lesional and nonlesional skin suggest that it might have a role in the pathogenesis of the disease.

Introduction

Haptoglobin (Hp) is one of the acute phase proteins (APPs), and its plasma level is raised by 2–4-fold during the inflammatory response.[1] Hp is synthesized primarily by the liver, but is also produced by other tissues including arterial vessels, skin, lung, spleen, brain, intestine and kidney.[2] The main function of Hp is to bind free haemoglobin (Hb) and transport it to the liver for degradation and iron recycling.[3] In addition to its role as an Hb scavenger, Hp has been shown to behave as an anti-inflammatory,[3,4] antioxidant[4] and angiogenic factor.[5] In addition, Hp has been reported to be a potent T-cell immunosuppressor.[6,7] We previously investigated the role of Hp in the pathogenesis of psoriasis,[8–10] and found that this protein displays some structural modifications potentially associated with protein function in the disease.[8] In addition, in a previous study, we found that Hp in patients with psoriasis had specific and more

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Conflict of interest: the authors declare that they have no conflicts of interest.

Accepted for publication 6 November 2014

abundant glycans compared with Hp from healthy controls, which might be associated with disease activity.[9] In the current study, we assessed the specific changes in Hp glycan structures associated with psoriasis, and the differences between circulating and cutaneous Hp.[10]

Psoriasis is an immune-mediated inflammatory disorder, characterized by epidermal proliferation, altered keratinocyte differentiation and inflammatory cell infiltrate.[11] In addition, substantial changes in Langerhans cell (LC) biology and function have been described.[12] These cells, retained within the epidermis, have been hypothesized to present antigen locally to sustain or exacerbate a cutaneous inflammatory reaction.[12] In psoriasis, a considerable impairment of LC mobilization is seen.[13] Indeed, several studies have suggested that UV-induced immunosuppression of epidermal LCs, together with the upregulation of T helper (Th)2 immunomodulatory cytokines, such as IL-10, which is enhanced in UVB-irradiated keratinocytes, might explain the therapeutic effects of phototherapy in psoriasis.[13,14] In addition, several studies performed by Schwarz *et al.* demonstrated that higher doses of UVB induced release of IL-10 from keratinocytes, and that IL-10 in turn caused inhibition of the antigen-presenting function of LCs,[14,15] while Xie *et al.*[16] reported that impairment of this function was possibly produced by Hp through inhibition of the functional maturation of LCs into antigen-presenting cells.

The aim of this study was to investigate the effects of UVB on Hp, in order to clarify the role of Hp in psoriasis.

Methods

The experimental protocol conformed to the principles of Helsinki Declaration, and was approved by the Ethics Committee of our Institution. Written informed consent was signed by all participants involved in the study.

Cell culture

HaCaT cells were cultured and irradiated with UVB 100 mJ/cm^2 as reported in our previous experiments.[17] After irradiation, cells were grown in Dulbecco modified Eagle medium (DMEM) without fetal bovine serum (FBS) for 6, 12, 24 or 48 h. mRNA extraction was performed at 6, 12 and 24 h, and protein collection was performed at 48 h. A nonirradiated plate was used as control.

RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted from HaCaT cells (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis; Roche, Indianapolis, IN, USA). Real-time quantitative reverse transcription (qRT-PCR) was used to analyse gene expression of Hp, IL-6 and IL-10. The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the 18S reference gene in the same sample. Fold induction of gene expression was calculated using the $^{2\Delta\Delta}$CT method.[18]

ELISA

Irradiated and control cells were cultured in six-well microtitre plates for 48 h and then lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) for ELISA. In detail, the wells were coated with 1 μg of rabbit anti-human Hp IgG (Sigma-Aldrich) in 50 μL of coating buffer (7 mmol/L Na_2CO_3, 17 mmol/L $NaHCO_3$, 1.5 mmol/L NaN_3, pH 9.6) overnight at 4 °C. Wells were then washed three times with TBS-T (130 mmol/L NaCl, 20 mmol/L Tris-HCl, 0.05% Tween 20, pH 7.4) and three times with high salt TBS (500 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4). Blocking solution in TBS (130 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4) containing 0.5% bovine serum albumin (Sigma-Aldrich) was added for 1 h at 37 °C. Cells were again washed with TBS-T, then loaded with cell lysates (50 μL) or different amounts of commercial Hp (Sigma-Aldrich; 0.5, 0.25, 0.125, or 0.05 ng in TBS-T) and incubated overnight at 4°C. Thereafter, wells were incubated with mouse antihuman Hp IgG (Sigma-Aldrich) and treated with goat antimouse horseradish peroxidase conjugated-IgG (Sigma-Aldrich). The lowest detection limit was 7.5 pg/mL.

Western blotting analysis

Hp detection in media samples from control or UV-treated cells was carried out by electrophoresis followed by immunoblotting. Aliquots of each supernatant were analysed and protein fractionation carried out for 1 h at 100 V. Following electrophoresis, the membrane was treated with 5% non-fat milk in TBS-T, washed with TBS-T, and incubated with rabbit anti-human Hp IgG (Sigma-Aldrich). Finally, the membrane was incubated with goat anti-rabbit IgG (Sigma-Aldrich). In the absence of a specific and well-accepted protein loading control for secreted protein, we used cell number and total protein for normalizing the total

amount of conditioned medium, as previously described by Lokeshwar *et al.*[19] The immune complexes were detected using an ECL detection system (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's protocol.

Skin biopsies

Skin biopsies were taken from lesional and nonlesional skin of 25 subjects with moderate to severe psoriasis after a 1-month wash-out of systemic treatment, and from 5 healthy controls (HCs).

Immunohistochemistry

Hp immunohistochemistry was carried out on lesional and nonlesional skin biopsies from patients with psoriasis and controls. Frozen sections were fixed with cold acetone. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions. Sections were stained with mouse anti-human Hp antibody (overnight at 4 °C) and then incubated with biotinylated secondary antibody. Peroxidase activity was revealed using diaminobenzidine (DAB) substrate (ImmPACT DAB, Burlingame, CA, USA). Counterstaining was performed with haematoxylin and immunohistochemistry staining intensity was assessed with TMARKER (http://comp-path.inf.ethz.ch) image analysis software,[20] which provides an algorithm that detects relevant cells in the image and calculates an estimate of the percentage staining on these cells only.

Statistical analyses

Statistics were performed with PRISM software (v4.0; GraphPad Software Inc. La Jolla, CA, USA). Normal data were analysed by the two-tailed *t*-test, and non-normal data with the Wilcoxon test. $P < 0.05$ was considered statistically significant.

Results

Ultraviolet B can modulate haptoglobin, interleukin-6 and interleukin-10 basal gene expression in HaCaT cells

Analysis of constitutive mRNA levels of Hp, IL-6 and IL-10 in HaCaT cells showed that Hp increased at 12 h and returned to basal levels by 24 h, whereas IL-6 and IL-10 gradually increased with a peak at 24 h. After UVB irradiation (100 mJ/cm^2), we observed an inversion of the trend for Hp and IL-6, plus a potentiating action on IL-10 (Fig. 1). Interestingly, Hp showed similar results to IL-10 after irradiation, whereas IL-6 followed a curve completely opposed to Hp. These results showed that UVB did have effects on Hp in HaCaT cells, suggesting that it might affect Hp and IL-10 comparably.

Ultraviolet B induces haptoglobin synthesis and secretion in HaCaT cells

Hp concentration in HaCaT cell lysates from UVB-treated and control cells was measured by ELISA, which showed that Hp was detectable only in samples obtained from cells after UVB treatment (Table 1). Western blotting to detect Hp in supernatants from irradiated and unirradiated HaCaT cells showed Hp only in supernatants of irradiated cells (Fig. 2). These data demonstrated that UVB did have effects on Hp synthesis and secretion in HaCaT cells.

Haptoglobin gene expression is increased in psoriatic skin

Hp mRNA was significantly higher in nonlesional and lesional skin of patients with moderate to severe

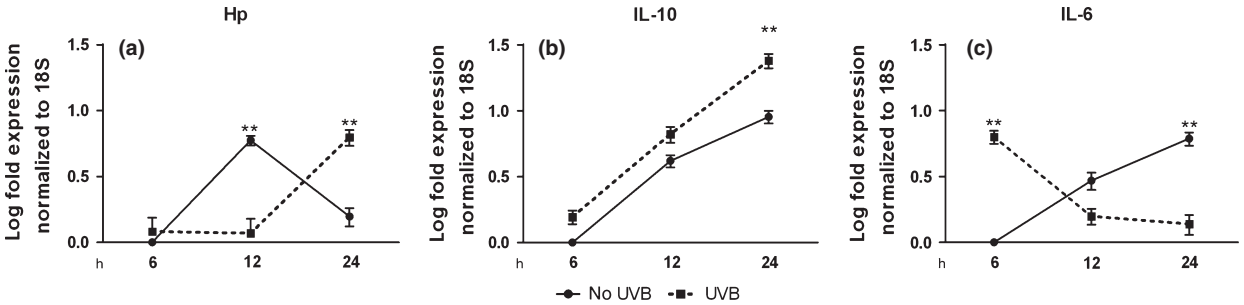

Figure 1 Haptoglobin (Hp), interleukin (IL)-6 and IL-10 mRNA levels were assessed in unirradiated HaCaT cells at 6, 12 and 24 h after ultraviolet (UV)B irradiation (100 mJ/cm^2) at the same time intervals. Data were normalized to the housekeeping gene 18S, and are presented as log expression compared with unirradiated HaCaT cells and expressed as means ± SD of three independent experiments. Statistical comparisons were performed using unpaired Student *t*-test; $^{**}P < 0.01$.

Table 1 Hp concentrations in whole-cell lysates from irradiated and unirradiated HaCaT cells cultured for 48 h and evaluated by ELISA.

HaCaT cells	Hp concentration, pg/mL
Irradiated	26.710 ± 0.003
Unirradiated	ND

ND, not detectable.
[Correction added after initial online publication on 11 May 2015: Hp concentration corrected for Irradiated and Unirradiated HaCaT cells.]

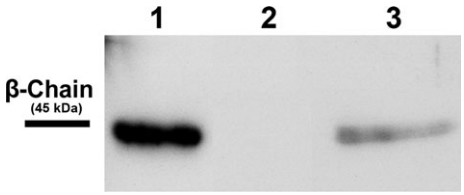

Figure 2 Western blotting of haptoglobin (Hp) was performed on supernatants from ultraviolet (UV)B (100 mJ/cm^2) irradiated (lane 3) and unirradiated HaCaT cells (lane 2) at 48 h. Lane 1, standard Hp. The picture is representative of data from three different experiments.

psoriasis compared with healthy skin from normal donors. No significant difference was seen between nonlesional and lesional skin of patients with psoriasis (Fig. 3). These findings confirm the enhanced gene expression of Hp in psoriatic skin, highlighting that a considerable increase, comparable with that in psoriatic plaques, was already present in unaffected skin.

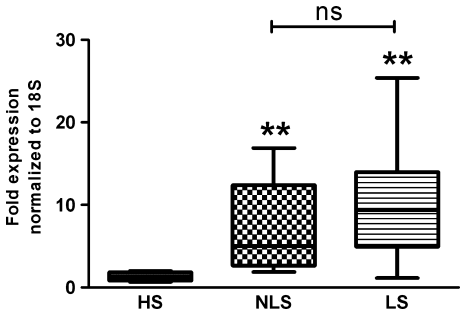

Figure 3 Hp gene expression was assessed in nonlesional and lesional psoriatic skin compared with healthy skin. Data, normalized to the housekeeping gene 18S, are displayed as boxes with the top and bottom representing the 25th and 75th percentiles, respectively; the line in the box represents the median, and whiskers represent minimum and maximum. Statistical comparisons were performed using Wilcoxon test. $^{**}P < 0.01$. HS, healthy skin; NLS, nonlesional psoriatic skin; LS, lesional psoriatic skin.

Table 2 Hp staining intensities evaluated by immunohistochemistry in different skin samples.

Skin samples	Hp staining, %*
HS	8.02 ± 1.38
NLS	20.15 ± 2.53†
LS	65.31 ± 4.06§

HS, healthy skin; LS, lesional skin; NLS, nonlesional skin. *Data are mean ± SD; †$P < 0.05$; §$P < 0.01$.
[Correction added after initial online publication on 11 May 2015: 'detection limits;' deleted from the table footnote.]

Haptoglobin protein is upregulated in psoriatic skin

Immunohistochemistry revealed Hp-positive staining in all three categories, with an increasing trend (Table 2). In particular, nonlesional skin showed positive cells mostly in the basal layers, similar to healthy skin but with a higher intensity, whereas lesional skin showed positive cells throughout the whole epidermis, with the main staining at the basal layers (Fig. 4). Notably, we observed Hp-positive staining of dendritic cells mostly in the suprabasal layers, which were most probably LCs.[16]

Discussion

Hp has been suggested to impair the immune response, but its role in psoriasis is still obscure. Abundance or structure of specific glycans, which are present in Hp from patients with psoriasis, and are different or missing in normal Hp, might be associated with disease activity. Our previous data demonstrated that specific changes in glycan structures of Hp, such as enhanced glycan branching and fucose content, are associated with psoriasis. Because very little is known about the effects of UVB on Hp, particularly on its glycan structures, we investigated Hp production in relation to UVB. We are also currently in the process of carrying out structural analysis to validate our hypothesis.

UVB irradiation can modulate skin immune responses through the secretion of various cytokines produced by keratinocytes, such as IL-6 and IL-10.[21] In the current study, we found that HaCaT cells showed a distinct curve of constitutive mRNA Hp expression with respect to IL-6 and IL-10. UVB produced an inversion of the Hp and IL-6 trend, as well as an enhancement of IL-10.

Interestingly, Hp showed similar results to IL-10 after irradiation, suggesting that UVB might affect Hp and IL-10 comparably. In addition, UVB induced production of Hp protein in HaCaT cells. These data indi-

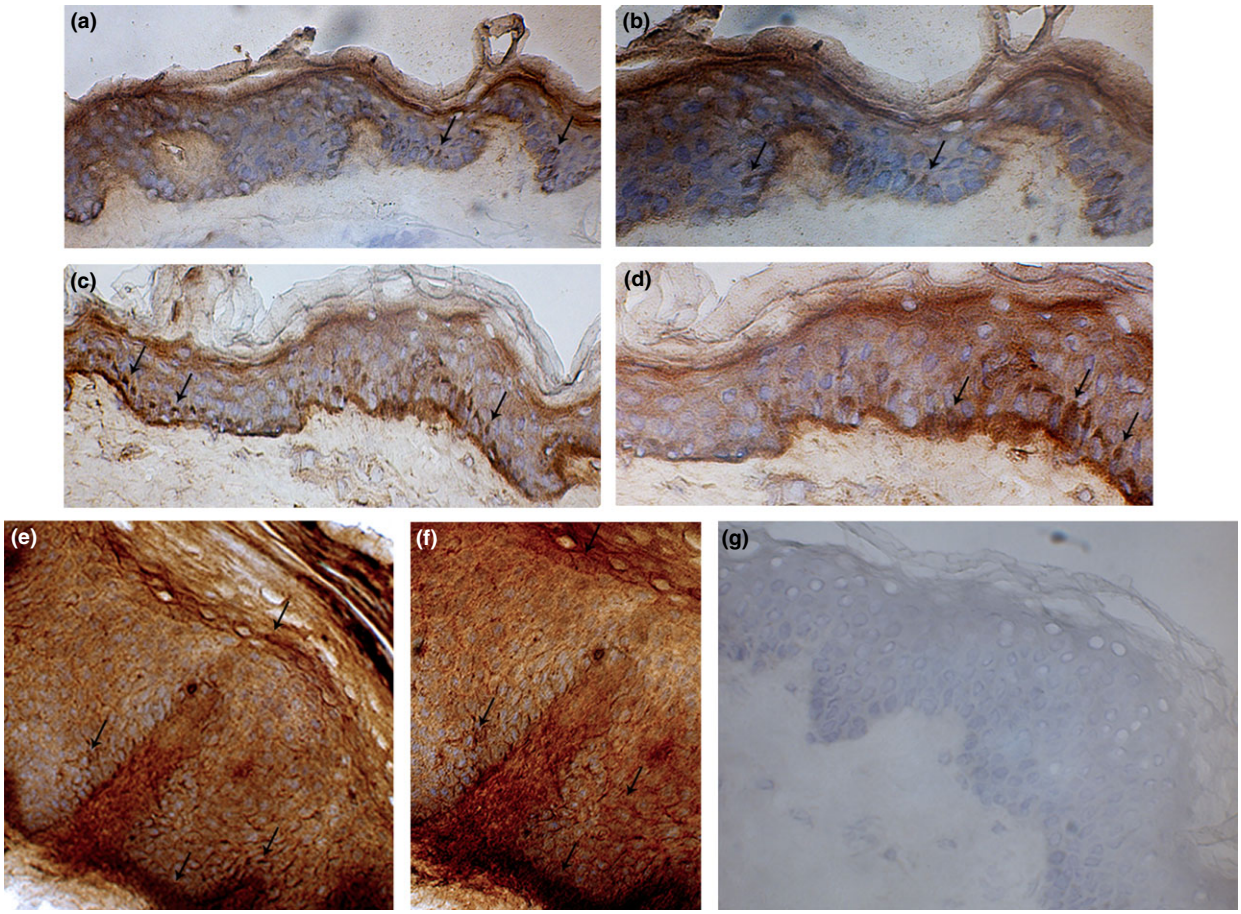

Figure 4 Haptoglobin (Hp) immunohistochemistry was performed in (a,b) healthy skin; (c,d) nonlesional and (e,f) lesional psoriatic skin, while (g) lesional psoriatic skin without primary antibody was used as control. Original magnification (a,c,e) × 250 and (d,f,g) × 400 magnifications.

cate a potential association between UVB and IL-6, IL-10 and Hp.

Upon UVB irradiation, keratinocytes express several pro-inflammatory cytokines, including IL-6. In the current study, IL-6, the earliest UVB-induced pro-inflammatory cytokine, was enhanced at 6 h after irradiation. The UVB-induced Hp increase occurred later, suggesting that IL-6 might stimulate Hp. Indeed, the secretion of Hp is enhanced in inflammatory states, and Hp has important anti-inflammatory properties.[3–5]

The Hb–Hp complex is removed from the circulation by binding to CD163, which is expressed by the monocyte–macrophage system. CD163 is a member of the scavenger receptor cysteine-rich family class B, and is expressed on most subpopulations of mature tissue macrophages. Triggering of CD163 by ligand binding (Hb–Hp) results in a protein tyrosine kinase-dependent signal and secretion of IL-6 and IL-10. In addition, IL-6 and IL-10 upregulate CD163, and this upregulation might function as a positive feedback mechanism for CD163 induction and activity. In addition, CD163 also exerts an immunomodulatory role by the degradation of heme, which results in the production of metabolites with suggested anti-inflammatory effects.[22]

It has been shown that Hp prevents spontaneous functional maturation of epidermal LCs in the skin.[16] Hp is stored in the epidermal LC cytoplasm, but LCs do not produce Hp; it is produced by keratinocytes. As a consequence, keratinocytes play an important role in regulating the function of LCs and T cells in the skin by producing cytokines and possibly by expressing Hp.[16,23]

Hp also has an important modulatory effect on the infiltration of mononuclear cells into the central ner-

vous system, and on the production of Th1 cytokines and IL-17A by autoreactive T cells. Exacerbated experimental autoimmune encephalitis in $Hp^{-/-}$ mice was shown to be related to increased expression of interferon-γ, IL-6 and IL-17A in the central nervous system.[24] These findings suggest that Hp has a protective role in reducing the severity of an autoimmune inflammatory process.

Hp has been described as an alternative low-affinity ligand for the CD11b/CD18 (Mac-1) integrin. As Mac1 is detectable on the cell surface of human resting LCs, our finding that Hp-positive cells are mostly localized in the suprabasal layers of the skin could be consistent with LC representation.[25] Interestingly, the increase in Hp-positive cells in nonlesional, but particularly in lesional psoriatic skin, supports the hypothesis that Hp might regulate differentiation and/or activity of T cells indirectly through the negative regulation of dendritic cell and macrophage functions in Th1/Th17 responses.[24]

What's already known about this topic?

- Hp has been shown to behave as an anti-inflammatory, antioxidant and angiogenic factor.
- We have previously investigated its role in psoriasis pathogenesis, showing that it displays some structure modifications associated with protein function in the disease.

What does this study add?

- This study showed that UVB irradiation was able to modulate Hp production *in vitro* in keratinocytes.
- In addition, high Hp *in vivo* levels in psoriatic skin, both lesional and nonlesional, suggest that it could have a role in the pathogenesis of the disease.

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