Possible role of BMP-4 in the hyper-pigmentation of psoriatic plaques after anti-TNF-α treatment

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Abstract. Psoriasis over-expresses several inflammatory mediators, which impacts the activity of melanocytes. Tyrosinase (Tyr) and microphthalmia-associated transcription factor (MITF) are the primary regulators of melanogenesis. Furthermore, bone morphogenetic proteins (BMPs) modulate various pathobiologic processes including inflammation, melanogenesis and melanomagenesis. To determine the association between psoriasis and melanogenesis, psoriatic lesional skin was screened through gene expression, immunohistochemistry, immunogold staining and melanin content assays. The present study detected a decreased expression of Tyr, MITF and BMP-4 in psoriatic lesional skin compared with healthy skin. Tyr, BMP-4 and melanin content were also evaluated in the psoriatic lesional skin of patients receiving adalimumab therapy, before and after 16 weeks of treatment. TNF-α blockade modulated the Tyr, BMP-4 and melanin content of the patient skin lesions, which supported the hypothesis that hyper-pigmentation may occur in areas of psoriatic plaque after biological treatment. The present study confirmed the influence of the psoriatic pro-inflammatory network on melanogenesis, exerting an inhibitory effect mediated by TNF-α. Furthermore, the results regarding BMP-4 in the present study add another important element to the mechanism of psoriasis.

Introduction

Psoriasis (PSO) skin over-expresses a myriad of inflammatory mediators [e.g., interleukin (IL)-1α, IL-6, IL-17 and tumor necrosis factor (TNF)-α], synthesized by different cell types, which dynamically interact with melanocytes (1-4). Synergistic action of these mediators down-regulates the pigmentation signaling pathway and melanin production (4-6). Previous studies have reported that primary human melanocytes respond to IL-17 and/or TNF-α stimulation forming clusters and modulating the expression of melanogenesis markers [e.g., microphthalmia-associated transcription factor (MITF) and tyrosinase (Tyr)] (7,8). Indeed, therapeutic neutralization of IL-17 and TNF-α with biologic agents is able to increase pigmentation signaling in the areas corresponding to the psoriatic plaques (8). However, the relationship between psoriasis and melanogenesis remains to clarify. Currently, melanocyte activity has been linked to the bone morphogenetic proteins (BMPs), a group of more than 20 secreted proteins also involved in the pathogenesis of PSO (9-11). In particular, melanin synthesis is up-regulated by BMP-2 and BMP-6, whereas controversial data exist on BMP-4 (5,11,12). Yaar *et al* (13) showed that BMP-4 supplementation of cultured human melanocytes decreased melanin synthesis. According to Cichorek *et al* (6), BMP-4 secreted by keratinocytes after ultraviolet (UV) radiation is able to increase melanogenesis. In the present study, we aimed to investigate the effect of psoriatic inflammatory network on melanogenesis uncovering a possible role of BMP-4 in this scenario.

Materials and methods

Study population. The overall study enrolment comprised 40 psoriatic and 40 healthy donors who had undergone plastic surgery. Psoriatic subjects were enrolled at the Dermatology out-patients clinic of the University of Naples Federico II whereas healthy ones were recruited at the Plastic Surgery Unit of the University of Naples Federico II. The study was approved by the Ethics Committee for Biomedical Activities 'Carlo Romano' of University of Naples Federico II, and conducted according to the Declaration of Helsinki principles. Each participant gave written informed consent before the onset of the study. Samples were collected between September 2017

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and June 2018. Patients and controls were similar to each other in terms of age (54±15 and 50±17, respectively) and male distribution (67.5 and 62.5%, respectively). Inclusion criteria for patients were: Diagnosis of moderate-to-severe PSO [Psoriasis Area Severity Index (PASI) >10], disease duration of at least 6 months, age ≥18 years, topical and/or systemic treatment washout period of at least 3 weeks, whereas for healthy subjects were: Age ≥18 years without a present- or past-positive history of PSO. Adalimumab (ADL) was administered subcutaneously 80 mg at week (W)-0 (baseline) to all psoriatic patients and successively 40 mg every other week, starting from W-1 and up to W-16. Lesional (LS) and non lesional skin (NLS) punch biopsies (3 mm diameter) were performed on trunk at weeks 0 and 16. Normal skin from plastic surgery remnants was used as control. Skin specimens were screened through gene expression, immunohistochemistry, immunogold staining and melanin content assay within 1 h of surgical intervention.

In vivo expression of Tyr, MITF and BMPs family members. RNA was extracted from skin biopsies (RNeasy Mini Protocol; Qiagen) and cDNA was prepared (Transcriptor High Fidelity cDNA Synthesis; Roche) according to the manufacturer's instructions. RT-qPCR (LightCycler; Roche) was used to analyze the levels of expression of 18S, Tyr, MITF, BMP-2, BMP-4, BMP-6, BMP-7. Relative mRNA levels were determined by the comparative threshold cycle method $2^{-\Delta\Delta cq}$ (14), and their expression was normalized to the expression of 18S mRNA as previously reported (15). PCR primers (18S, Tyr, MITF, BMP-2, BMP-4, BMP-6, BMP-7) were designed based on published sequences, and their specificity was verified with BLAST alignment search. To confirm amplification of the expected size fragment, amplification products were characterized by agarose gel electrophoresis. Melting curve analysis was carried out after completion to confirm the presence of single amplified species.

Ex vivo expression of Tyr and BMP4. Full-thickness skin, normal human epidermal sheets and dermis were obtained from healthy donors, and stimulated with recombinant human TNF-α protein (R&D Systems) at 20 ng/ml for 24 h. Next, samples were snap-frozen in liquid nitrogen and stored at -70˚C until RNA extraction. Major details are reported in supplementary materials (Appendix S1).

Immunohistochemistry. The immunohistochemical detection of Tyr and BMP-4 was carried out on LS samples of 10 psoriatic patients before (baseline) and after 16 weeks of ADL therapy. Healthy skin samples were used as controls. Specimens were immediately placed in tissue freezing medium (Jung; Leica) and stored at -80˚C. Five micrometer sections were cut with a cryostat and fixed with cold methanol for 10 min. The Vectastain Elite ABC Kit (Vector Laboratories) was used as follows: Sections were incubated with blocking solution [horse serum diluted in buffer: Phosphate buffered saline (PBS) + bovine serum albumin 1%] for 20 min at 22˚C. Biopsies were stained with anti-tyrosinase (1 μg/ml; Gibco), anti-BMP-4 (10 μg/ml; Fitzgerald) and incubated overnight at 4˚C. In parallel, skin specimens were incubated with specific isotype control antibodies (Mouse IgG1 Isotype Control, Mouse IgG2B Isotype Control, Goat IgG Control; R&D System) used at the same concentration as the corresponding primary antibody. The sections were then washed in buffer and incubated with biotinylated secondary antibody for 30 min at room temperature. Peroxydase activity was revealed using DAB substrate (ImmPACT DAB). Counterstaining was performed with hematoxylin. Staining was observed using Nikon Eclipse E600 epifluorescence microscope (Nikon).

Immunogold staining. Tissue samples were fixed in a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde in PBS overnight at 4˚C and washed in the same buffer. Following dehydration, samples were embedded in Epon resin, and ultra-thin sections (60 nm) were collected on 200 mesh nickel grids. Samples were washed 3 times in distilled water for 5 min, equilibrated in PBS containing 1.5% goat serum and 1% BSA for 15 min and incubated overnight at 4˚C with anti-Tyr (1 μg/ml; Gibco), anti-BMP-4 (10 μg/ml; Fitzgerald) diluted in PBS/BSA 1%. Following 5 washings (2 min each) in PBS and 5 washings in PBS/BSA 0.5%, sections were incubated in 1% PBS/BSA for 15 min and then for 1 h at room temperature with the goat anti-mouse secondary antibody (H&L) labeled with 20-nm gold particles (BB International). Sequential washings were performed in 1% PBS/BSA for 15 min, in 0.5% PBS/BSA 5 times for 2 min, in PBS 5 times for 2 min, and in distilled water twice for 1 min. After staining with uranyl acetate, sections were analyzed using a Leo 912AB electron microscope (Carl Zeiss). Controls, based on the use of only secondary antibody, were run in parallel.

Melanin content assay. Total cellular melanin content was performed in healthy and psoriatic skin biopsies before and after 16 weeks of ADL treatment using Fontana-Masson staining (AMTS Inc.) according to the manufacturer's instructions.

Statistical analyses. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc.). The Kruskall Wallis and Mann-Whitney tests were used for all intergroup comparisons followed by post hoc corrections for multiple comparisons using Bonferroni test. Wilcoxon test was used for paired samples. Values of $P<0.05$ were considered significant and all data were displayed as means ± standard deviation (SD).

Results

To better explore the link between psoriatic inflammation and melanogenesis, we examined gene expression of Tyr, MITF and some members of BMPs family (BMP-2, BMP-4, BMP-6 and BMP-7) in PSO LS and NLS compared to healthy skin (HS). Our results showed that Tyr and MITF were decreased in PSO LS compared to HS (Fig. 1A and B). Likewise, BMP-4 and BMP-7 were found significantly reduced in PSO LS (Fig. 1D and F), whereas no significant difference was observed for BMP-2 and BMP-6 (Fig. 1C and E).

To verify the effects exerted by anti-TNF-α therapy on melanogenesis markers in psoriatic lesions, we evaluated

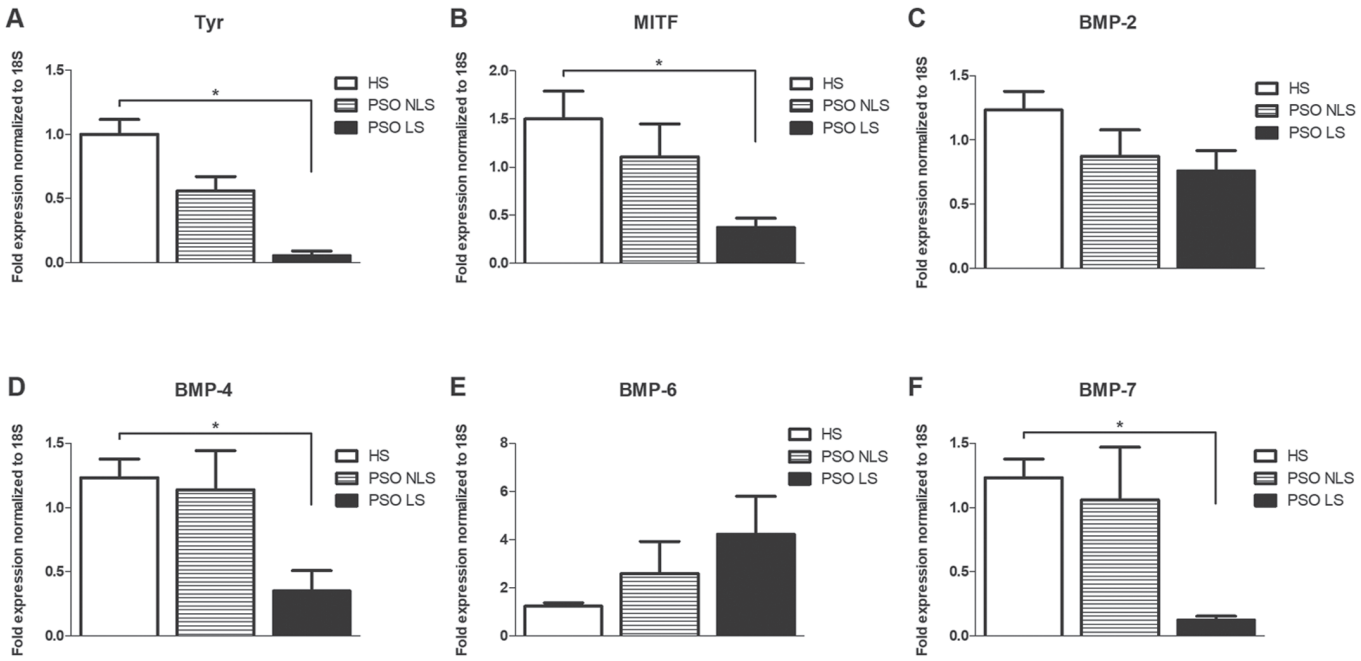

Figure 1. Melanogenesis markers and BMP family members are differentially expressed in HS, PSO LS and NLS. Gene expressions of (A) Tyr, (B) MITF, (C) BMP-2, (D) BMP-4, (E) BMP-6 and (F) BMP-7 are presented. Data are presented as the mean ± standard deviation. *P<0.05 as indicated. Tyr, tyrosinase; MITF, microphthalmia-associated transcription factor; BMP, bone morphogenetic protein; HS, healthy skin; PSO, psoriasis; LS, lesional skin; NLS, non lesional skin.

Tyr, BMP-4 and melanin content in PSO LS before and after 16 weeks of ADL treatment. Patients who did not achieve a 50% PASI reduction at W-16 were defined as non responders. Immunohistochemical analysis revealed that both Tyr (Fig. 2A) and BMP-4 (Fig. 2B) were lower in PSO LS (W-0) compared to HS, confirming gene expression analysis. At W-16, Tyr (Fig. 2A) and BMP-4 (Fig. 2B) were enhanced in the lesions of responder patients respect to W-0, whereas a lower intensity of both markers was still observed in non responder subjects (Fig. 2A and B). The ability of TNF-α to modulate Tyr and BMP-4 was further confirmed by *ex vivo* experiments (Fig. S1). In particular, TNF-α was able to reduce Tyr expression in normal human epidermal sheet and HS organ culture, whereas a significant reduction of BMP-4 was confirmed just in normal human epidermal sheet (Fig. S1A-D). More evidence on Tyr and BMP-4 in PSO LS was obtained through immunogold staining. Both markers were lower in keratinocytes of PSO LS compared to HS (Fig. 2C). Melanin content produced by melanocytes was greater in PSO LS (W-0) than HS (Fig. 2D). After 16 weeks of ADL therapy, melanin was reduced in the lesional skin of responders respect to baseline (W-0) (Fig. 2D), whereas no melanin reduction was observed in non responder plaques (Fig. 2D). The phenomenon of hyper-pigmentation was clinically evident in responder patients at W-16 (Fig. 2E).

Discussion

Pigmentary changes in psoriasis may be modulated by a myriad of inflammatory mediators that are overexpressed in psoriatic lesions. Some of these mediators are known to have hypo-pigmenting effects (e.g., IL-1α, IL-6, IL-17, TGF-β1, TNF-α), and can independently regulate the expression of MITF, Tyr and related enzymes (4,6,7). In the present study, we have reported that MITF and Tyr were significantly decreased in psoriatic lesions with respect to healthy skin. Our data are consistent with research of Wang (7). The authors showed a reduction of main melanogenesis markers in contrast to a higher presence of melanocytes in psoriatic lesions. Apart from a decrease of Tyr, we observed a reduction of BMP-4 in psoriatic plaques. Moreover, an increase of Tyr and BMP-4 was assessed in responder patients after 16 weeks of anti-TNF-α therapy, whereas levels similar to baseline were encountered for non responder subjects. As hypothesized by Di Cesare *et al* (8), high levels of melanogenesis markers induced by the treatment with TNF-α blockers could increase pigmentation signaling pathway. A possible role of BMP-4 in psoriatic post-inflammatory hyper-pigmentation has not been explored, even though controversial data exist on this marker in the process of melanogenesis (6,13). We have found that BMP-4 is linked to Tyr given that they shared the same trend in response to anti-TNF-α treatment. Thus, this evidence supports that BMP-4 is able to increase melanogenesis as reported by Cichorek *et al* (6). In particular, BMP-4 released by keratinocytes acts as a paracrine factor that directly influences melanocytes activity.

It has to be taken into account that we have found a decrease of melanocytes number upon ADL treatment. This might be in contrast with post-inflammatory hyper-pigmentation, but the concomitant increase of Tyr as well as BMP-4 may render local melanocytes more active in the melanogenesis process. Taken together, the innovative and intriguing results on BMP-4 sheds the light on its possible involvement in hyper-pigmentation phenomenon in the areas corresponding to psoriatic plaques during anti-TNF-α therapy. Further studies on BMP-4 in this scenario would be valuable.

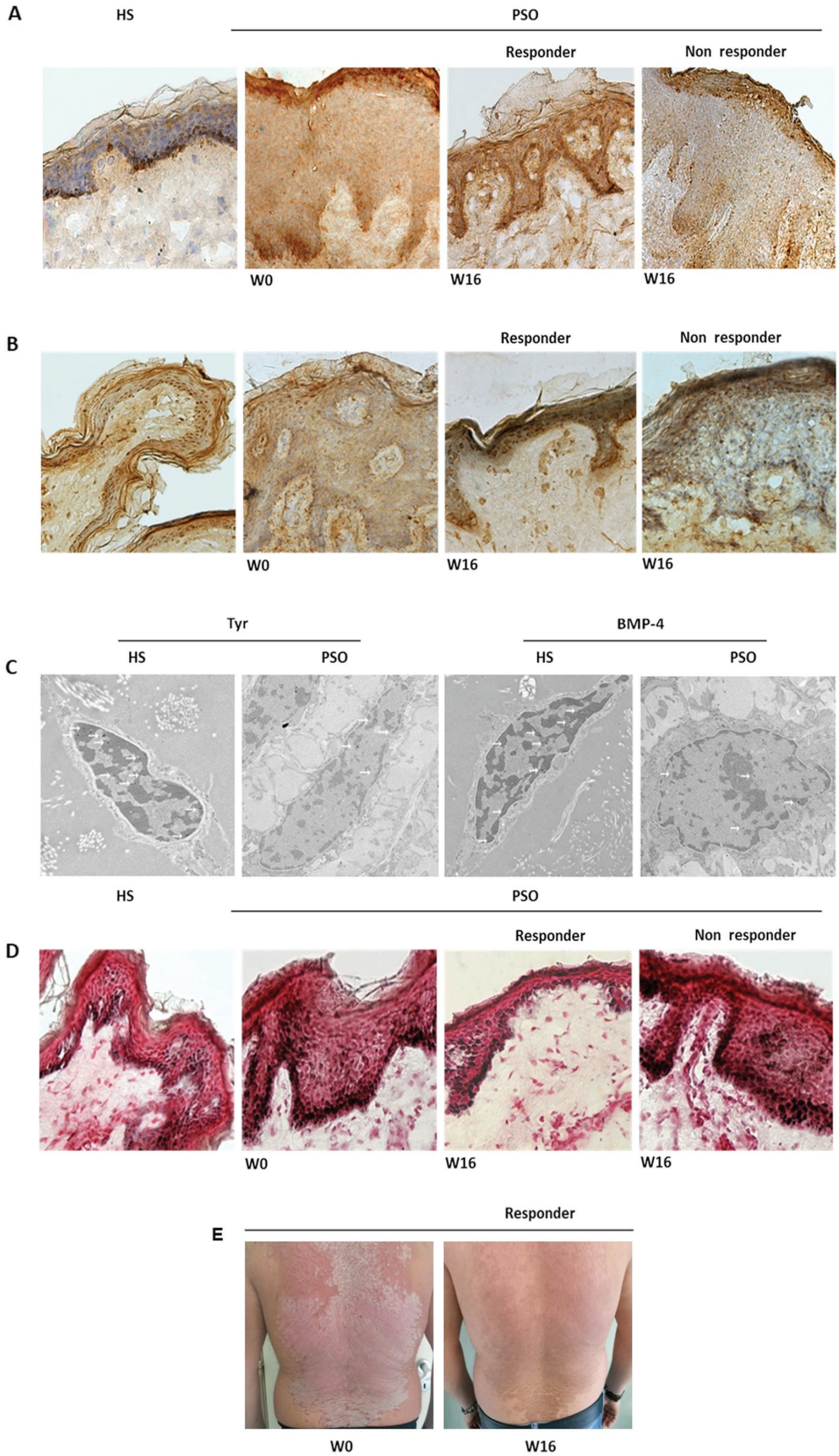

Figure 2. Tyr, BMP-4 and melanin levels are modulated by anti-tumor necrosis factor-α therapy in psoriasis. Immunohistochemical detection of (A) Tyr and (B) BMP-4 in HS and PSO LS samples before and after 16 weeks of adalimumab therapy. Lower intensities of Tyr and BMP-4 were detected in PSO LS at W0 compared with HS. At W16, Tyr and BMP-4 were higher in the LS of responder patients, whereas both markers remained low in lesions of non-responder patients (magnification of each, x20). (C) Immunogold staining in HS and PSO LS. BMP-4 and Tyr were detected at the keratinocyte level (white arrows). Reduced marker levels were detected in PSO LS compared with HS (magnification, x5000). (D) Melanin content assay in HS and PSO LS before and after 16 weeks of adalimumab therapy. At W0, a greater content of melanin was exhibited in psoriatic skin when compared with HS. At W16, melanin was reduced in the lesional skin of responders when compared with W0, whereas no melanin reduction was observed in non-responder plaques (magnification, x20). (E) Clinical manifestation of psoriasis at W0 and after 16 weeks of adalimumab therapy. Representative image of a responder patient at W16 exhibiting post-inflammatory hyper-pigmentation in areas of plaque. Tyr, tyrosinase; BMP, bone morphogenetic protein; W, week; PSO, psoriasis; LS, lesional skin; HS, healthy skin.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LDC, ES, GC, SL, RM, MM, AP, RDC and AB conceived the current study, acquired, interpreted and analyzed the data, and drafted the manuscript. LDC, ES, SL and AB revised the manuscript for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the study.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Biomedical Activities 'Carlo Romano' University of Naples Federico II (Protocol no. 160/010) and is in accordance with the legal requirements of the Declaration of Helsinki. Each patient provided written informed consent prior to enrolment.

Patient consent for publication

Consent for the publication of images was obtained from patients included in the present study.

Competing interests

The authors declare that they have no competing interests.

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