INVESTIGATIVE REPORT

Increased Expression of the Wnt Signalling Inhibitor Dkk-1 in Non-lesional Skin and Peripheral Blood Mononuclear Cells of Patients with Plaque Psoriasis

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Psoriasis is a common inflammatory skin disease characterised by abnormal keratinocyte proliferation, increased dermal angiogenesis and systemic inflammation. The cell signalling cascades provoked by Wnt proteins and their inhibitors, such as Dickkopf-1 (Dkk-1), play crucial roles to maintain homeostasis of a variety of tissues, including skin, and are also involved in angiogenesis and innate immunity. This study was designed to investigate the distribution of Dkk-1, in lesional and non-lesional skin, in serum and in peripheral blood mononuclear cell (PBMCs) of patients with psoriasis compared with healthy controls. Our results showed significantly increased mRNA and protein expression of Dkk-1 in non-lesional compared with lesional skin and healthy control skin. No significant differences of Dkk-1 serum levels were observed, but Dkk-1 protein expression was significantly increased in patients' PBMC. Increased levels of Dkk-1 in PBMC, suggest a possible role of Dkk-1 in the chronic systemic inflammation of psoriasis. Increased levels of Dkk-1 in non-lesional psoriasis skin offers new insights in the local inflammatory processes in psoriasis skin since Wnt signalling regulates angiogenesis. In conclusion, Dkk-1 may be a possible target for future treatment options. *Key words: psoriasis; Dickkopf-1; Wnt-proteins; peripheral blood mononuclear cells.*

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The Wnt family of glycoproteins are well conserved through evolution and they are involved in multiple cellular activities that are crucial for cellular functions, such as growth, differentiation, and death (1). Recent reports suggest a role of Wnt proteins in inflammation and in human immune defence against infections (2, 3). There are currently 19 Wnt genes identified and Wnt signalling has been divided into 2 major pathways. The canonical signalling pathway involves Frizzled receptors and low-density lipoprotein receptor-related proteins (LRPs) 5 and 6 as co-receptors (4). This pathway activates nuclear translocation of β-catenin and is typically linked to cell-fate deter-mination and stem cell maintenance. The non-canonical signalling pathway is independent of β-catenin activation and involves Frizzled receptors (5). Wnt antagonists can be divided into 3 functional classes: the Frizzled-related protein class, the Dickkopf class and Wnt inhibitory factor (WIF) (6, 7). Members of the Dickkopf class, such as Dickkopf-1 (Dkk-1) are secreted proteins inhibiting Wnt signalling by interacting with the LRP5/LRP-6 component of the Wnt receptor complex (8). Antagonists of the Dickkopf class specifically inhibit the canonical Wnt pathway.

Psoriasis is characterised by chronic systemic inflammation associated with activated innate immune pathways and altered differentiation and hyperproliferation of keratinocytes (9). In the light of the fundamental roles of Wnt proteins in controlling cell proliferation and differentiation and a potential role in innate immunity surprisingly little is known about the expression and the potential function of Wnt homologues and their antagonists under pathophysiologic conditions, such as in psoriasis. Beta-catenin, the transcription factor for Wnt signalling, is increased in lesional suprabasal psoriatric epidermis (10). Wnt16B has a role in mediating keratinocyte proliferation (11) and Dkk-1 regulates skin pigmentation and thickness by affecting Wnt-signalling (12). Two reports describe increased expression of Wnt5a mRNA and protein in lesional skin of patients with psoriasis (13, 14).

The aim of this study was to describe the expression of the Wnt antagonist Dkk-1 in non-lesional and lesional skin of patients with psoriasis compared to healthy controls and to analyse the expression of Dkk-1 in peripheral blood mononuclear cells (PBMCs) and serum.

MATERIAL AND METHODS

Study subjects

In all, 47 patients with mild to severe plaque type psoriasis and 25 normal healthy controls were enrolled for the study (male/female 29/18 and 7/18; mean ± SD age 55.3 ± 14.6 and 49.2 ± 17.0, respectively). Written informed consent was obtained from all subjects under protocols approved by the ethical committee Linköping University, Linköping, Sweden. This study was conducted in compliance with good clinical practice and according to the Declaration of Helsinki Principles. Sex, age and disease duration were recorded, as well as an assessment of disease severity using the Psoriasis Area and Severity Index

(PASI) showed mean $\pm$ SD of 7.1 ± 6.5. Full-thickness punch biopsies were taken from non-lesional skin (with a distance of at least 10 cm from any psoriatic lesion; 4 mm diameter) and from the active margin of a psoriatic plaque (4 mm diameter) from every patient. Study subjects did not use any systemic anti-psoriatic treatments for 2 weeks prior to biopsy. One biopsy was obtained from corresponding anatomical sites from healthy controls. Immediately upon removal, biopsies were stored either in formalin for immunohistochemistry or RNA later for gene expression analysis (Ambion, Austin, USA) and stored at –80°C.

Blood samples were taken from patients with psoriasis and controls for gene expression analysis from peripheral blood (Tempus Blood RNA tubes, Life technologies corporation, Carlsbad, CA, USA) and Dkk-1 protein analysis in serum and from PBMCs. Blood samples were immediately stored at –80°C prior to subsequent analysis.

Immunohistochemistry

Immunohistochemistry of paraffin-embedded 3.5 µm tissue sections from healthy control subjects ($n = 10$), non-lesional ($n = 11$) and lesional ($n = 11$) skin from patients with psoriasis was performed. Sections were placed on slides with adhesive coating and then dried in hot chamber at 59–60°C overnight. After deparaffinisation slides were pretreated in buffer Diva (Biocare Medical, Concord, USA) in a pressure cooker, maximum heating 110°C, for antigen retrieval. Immunohistochemical staining was performed by a polymer peroxidase method, MACH4 (Biocare Medical, Concord, CA, USA). Visualisation was completed by diaminobenzidine and counterstaining by haematoxylin Tachas (Biocare Medical, Concord, CA, USA). As primary antibody anti-Dkk-1 mouse monoclonal IgG antibody (0.5 mg/ml, Nordic BioSite, Stockholm, Sweden) was used. Isotype control and a negative control using PBS instead of primary antibody were performed to check for nonspecific binding and false positive results. Positive control was performed with placenta tissue known to express high levels of Dkk-1. Stained sections were examined by light microscopy, and from each stained tissue section 3 digital images with a 20 X objective were taken. Immunohistchemistry digital images were used for quantitative analysis using Picsara software (Vers. 9.4, Euromed, Stockholm, Sweden). Briefly, the pixel intensity of separated DAB staining was analysed and the number of pixels with the same specific intensity was calculated in the entire digital image including dermis and epidermis. Immunostaining intensity per unit area (positive pixels per 100,000 pixels) was calculated in each digital image and the mean intensity in 3 different images per case was calculated.

RNA purification and gene expression analysis

RNA was purified according to the manufacturer's instructions. Briefly, biopsies were homogenised using a TissueRuptor and disposable probes (Qiagen, Hilden, Germany), and RNA was purified using the AllPrep DNA/RNA mini kit (Qiagen) and the automated QIAcube system (Qiagen). RNA from stabilised blood was purified using the Tempus Spin RNA Isolation Reagent kit (Life technologies). Concentration and purity was measured using a Nanodrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and RNA integrity was assessed using the RNA integrity number with a 2100 Bioanalyzer (Agilent technologies, Santa Clara, USA).

RNA was reverse transcribed using the High capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies), according to the manufacturer's instructions, and the resulting cDNA was stored at –80°C.

Expression of *DKK1*, and of 2 housekeeping genes, *ACTB* and *GAPDH*, were analysed on the 7500 Fast real-time PCR system (Applied Biosystems, City, Country) and the standard run mode using Taqman Gene Expression Assays (Hs00183740_m1, Hs99999903_m1 and Hs03929097_g1, respectively) and TaqMan Universal Master Mix no UNG (Applied Biosystems). For each assay and sample, cDNA based on 10 ng were analyzed in a total volume of 20 µl. Threshold cycle (C_T) values were established using the ExpressionSuite software version 1.0.1 (Life Technologies). Missing CT values, due to low copy numbers, were replaced by the highest C_T value available for the gene in question, increased by one cycle. For the target gene (*DKK1*) the resulting CT values were normalised using C_T values of the selected reference genes, *GAPDH* in blood and *ACTB* in skin biopsies.

ELISA

Dkk-1 concentration in serum and in peripheral blood mononuclear cells was measured according to the manufacturer's instructions using a commercially available ELISA kit (Quantikine, Human Dkk-1 Immunoassay, R&D Systems, Minneapolis, USA). Duplicate 100 µl serum samples and 100,000 PBMCs after repeated freezing and thawing cycles were added to the assay.

Statistics analysis

Statistical evaluation of multiple groups was performed by Kruskal-Wallis ANOVA by Ranks and Mann-Whitney *U* test as a *post hoc* test and Student's *t*-test was used to compare 2 groups. All statistical analysis was performed using Statistica 12 software (Statistica, Tulsa, OK, USA). All $p < 0.05$ were considered significant.

RESULTS

In normal skin of healthy controls immunohistochemistry showed weak epidermal staining of Dkk-1 and staining of endothelial cells and fibroblasts (Fig. 1A). In non-lesional psoriasis skin epidermal staining of Dkk-1 was more intense and prominent at the epidermal basal cell layer (Fig. 1B). In lesional psoriasis skin epidermal Dkk-1 staining was absent and intense positive Dkk-1 staining of the inflammatory perivascular infiltrate was present (Fig. 1C). Quantification revealed that Dkk-1 immunostaining signal (dermal and epidermal) in non-lesional skin of patients with psoriasis was significantly increased compared with lesional skin and control subjects (Fig. 2A).

Dkk-1 mRNA expression was significantly increased in non-lesional skin from patients with psoriasis compared with lesional skin and control samples (Fig. 2B).

Analysis of PBMCs showed increased expression of Dkk-1 protein in patients with psoriasis compared to controls ($p < 0.01$, Fig. 3).

Our data revealed no significant differences in serum levels of Dkk-1 protein or in Dkk-1 gene expression in PBMC of patients with psoriasis and controls (Fig. S1[1]).

DISCUSSION

Abnormal Wnt signalling has been associated with many human diseases, ranging from cancer to degenerative

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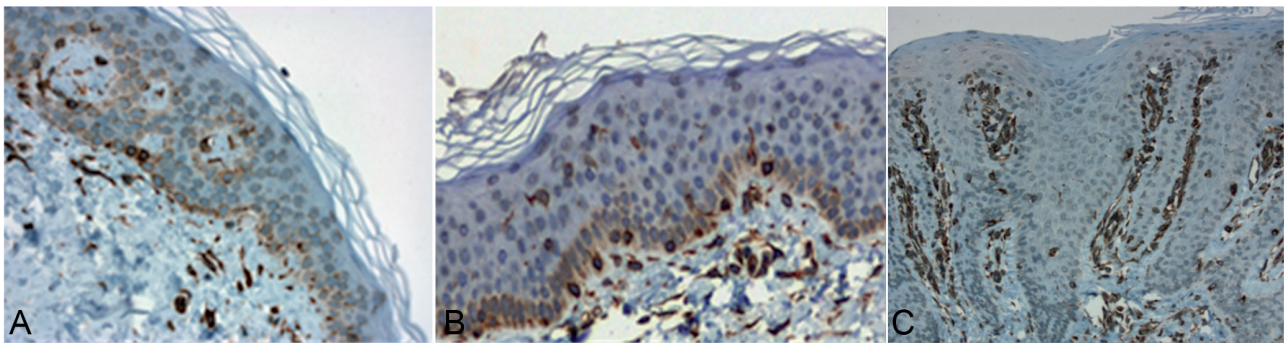[1]https://doi.org/10.2340/00015555-1970

Fig. 1. Expression of Dkk-1 protein (brown) in skin from healthy controls (A), in non-lesional (B) and lesional skin (C) from patients with psoriasis analysed by immunohistochemistry. 20× magnification (A and B) and 10× magnification (C).

disorders. The Wnt pathway has also been suggested to play a distinct role in inflammation and in linking innate and adaptive immunity to infections (2, 15). In the present study we report increased gene and protein expression of Dkk-1, a regulatory molecule of the Wnt pathway, in non-lesional skin of patients with psoriasis compared with lesional skin and healthy controls. Wnt signalling is known to induce angiogenesis by increased synthesis of vascular endothelial growth factor (VEGF) (16). Increased Dkk-1 expression in non-lesional psoriatic skin may inhibit angiogenesis by antagonising Wnt signalling thus sustaining the clinical picture of uninvolved skin. Owing to the significance of Wnt signalling in angiogenesis, Wnt antagonists, such as Dkk-1 have been considered potential treatments for neovascular disorders (17). Interestingly, *DKK-1* is a pro-apoptotic gene and may play an important role in connecting the oncogenic Wnt and p53 tumour suppressor pathways (18). The pro-apoptotic effect of Dkk-1 may lead to sustained skin homeostasis in clinical non-lesional psoriatic skin and decreased Dkk-1 expression in psoriasis skin may therefore contribute to pathological activation
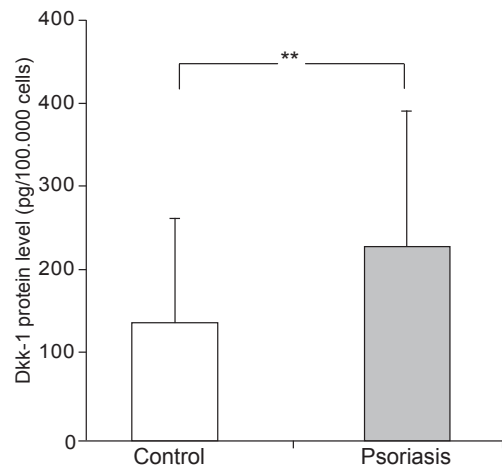

Fig. 3. Significant increased expression of Dkk-1 protein in peripheral blood mononuclear cells of patients with psoriasis (**$p < 0.01$, controls $n = 15$, psoriasis $n = 45$).

of canonical Wnt signalling. Epidermal Langerhans' cells (LCs) have pivotal roles in initiating immunity by acquiring antigens that are encountered in skin. Recent research showed that Dkk-1 reduces LC proliferation in mice (19). It is tempting to speculate that increased Dkk-1 in non-lesional psoriatic skin may inhibit LC proliferation and hence diminish inflammation in psoriasis; but further studies are needed to test this hypothesis.

PBMCs are main actors in inflammatory processes and they are linked to many diseases, such as atherosclerosis and psoriasis (20–22). Our study revealed significantly enhanced levels of Dkk-1 protein in PBMCs of patients with psoriasis. Recent findings identify Dkk-1 as a novel mediator in platelet-mediated endothelial cell activation and Ueland et al. (23) showed higher Dkk-1 expression in carotid plaques suggesting a role for Dkk-1-mediated inflammation in atherosclerotic lesions. In the light of these findings it would be interesting to
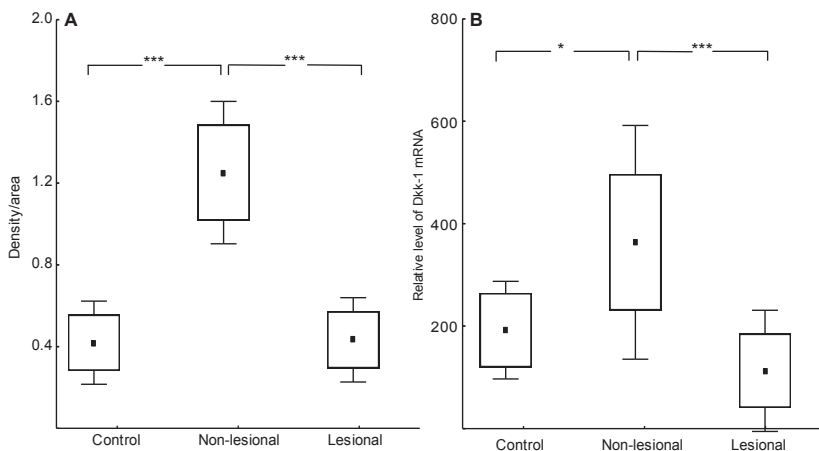

Fig. 2. (A) Increased expression of Dkk-1 protein in non-lesional skin from patients with psoriasis compared with lesional skin and skin from healthy controls (controls $n = 10$, psoriasis $n = 11$). (B) Increased Dkk-1 gene expression in non-lesional skin from patients with psoriasis compared with lesional skin and skin from healthy controls (controls $n = 21$, psoriasis $n = 31$). (box = mean ± 0.95 confidence interval, whiskers = mean ± SD, * $p < 0.05$, *** $p < 0.001$).

further investigate the role of increased Dkk-1 PBMC levels in the increased cardiovascular risk profile of patients with psoriasis. Activation of the innate immune system is a key event in the early steps of psoriasis. Interestingly, recent data link Wnt signalling to innate immune functions. Wnt5a and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation suggesting a role of Wnt proteins in human immune defence against infections (2). Further studies are needed to elucidate the effect of increased Dkk-1 in PBMCs on innate immunity and psoriasis.

Treatment of ankylosing spondylitis and rheumatoid arthritis with TNF-α inhibitors was accompanied by decreased Dkk-1 serum levels (24, 25). These studies emphasise Dkk-1 as a protagonist in chronic immune mediated diseases and Dkk-1 may serve as a biomarker for the activity of these diseases. This is less likely to be the case for psoriasis since our data did not reveal a significant difference in serum levels between patients and controls. However, the serum results are not concordant with the observed increased Dkk-1 levels in non-lesional skin and in PBMCs of patients with psoriasis. This discordance might be due to low serum secretion and intracellular storage of Dkk-1 or post translational modifications of secreted Dkk-1 complicating serum detection.

In conclusion, the present study describes alterations in the expression of Dkk-1 in patients with psoriasis compared to controls, yet the role of Dkk-1 in the complex immune mediated pathogenesis of psoriasis is still unclear. Investigating the effect of Dkk-1 substitution to psoriasis skin or inhibiting Dkk-1 in PBMCs has to show whether or not Dkk-1 plays a role in the development of psoriasis.

ACKNOWLEDGEMENTS

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