Differential effects of Wnt5a on the proliferation, differentiation and inflammatory response of keratinocytes

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Abstract. The predominant role of Wnt family member 5A (Wnt5a) is to induce non-canonical Wnt signalling pathways, including the Wnt-Ca\(^{2+}\) and Wnt-planar cell polarity pathways. Enhanced Wnt5a expression is involved in the formation of psoriatic plaques; however, its mechanistic role remains to be determined. In the present study, the effects of Wnt5a expression on HaCaT keratinocytes were investigated. HaCaT cells were cultured in medium supplemented with 0, 40 or 80 ng/ml Wnt5a for 24 h. Cell proliferation, the cell cycle, gene expression and inflammatory responses were investigated using Cell-Counting Kit-8 assays, flow cytometry analyses, reverse transcription-quantitative polymerase chain reaction analyses and enzyme-linked immunosorbent assays, respectively. Wnt5a treatment was revealed to suppress cell proliferation in HaCaT cells. Furthermore, Wnt5a was also demonstrated to increase the proportion of HaCaT cells arrested at the G2/M phase of the cell cycle, but reduce the proportion of HaCaT cells arrested at G0/G1 phase cells. In addition, the expression levels of the differentiation markers, including filaggrin, keratin 1 and keratin 10 were revealed to be downregulated in HaCaT cells. Expression of the canonical Wnt signalling genes (\(\beta\)-catenin and cyclin D1) and proliferation markers, such as Ki-67 and proliferating cell nuclear antigen in HaCaT cells were also revealed to be downregulated. However, the expression levels of inflammatory response markers (interferon-\(\gamma\), interleukin-8 and interleukin-17A) were revealed to be upregulated in HaCaT cells following Wnt5a treatment. These findings suggest that Wnt5a expression may be involved in the inhibition of cell differentiation and the induction of an inflammatory response in patients with psoriasis.

Introduction

Psoriasis is a chronic and systemic inflammatory disease, with an estimated population prevalence of 2-5% worldwide (1,2). Psoriasis is characterized by red, demarcated skin lesions with adherent silver scales (2). The scales are formed by hyperproliferative keratinocytes. In psoriatic skin, the fraction of proliferating keratinocytes is ~100%, whereas in normal skin it is ~20% (3). The epidermal cell turnover in psoriasis is increased 8-fold compared with normal skin, which may lead to altered keratinocyte differentiation (1,3,4). The increased rate of keratinocyte proliferation may be induced by cytokines secreted by activated resident immune cells, keratinocytes themselves; or by infiltrating inflammatory cells, including T cells, dendritic cells (5,6). Keratinocytes can respond to dendritic cell-derived and T cell-derived cytokines, including interferon (IFN), tumour necrosis factor (TNF), interleukin (IL)-17, and the IL-20 family. Furthermore, these T cell-derived cytokines can then produce pro-inflammatory cytokines (IL-1, IL-6, TNF-\(\alpha\), as well as chemokines such as IL-8, C-X-C motif chemokine 10, chemokine (C-C motif) ligand 20 (6).

The Wnt protein family is involved in cell proliferation, differentiation, polarity, adhesion and motility (7). Wnt family member 5a (Wnt5a) is one of the most extensively studied of the Wnt protein family members and represents a prototypical non-canonical Wnt family member (7). Wnt5a has a complex biological activity and can positively or negatively affect cell proliferation in different cell types (8,9). In addition, Wnt5a can both activate and inhibit the canonical Wnt signalling pathway (10,11). A previous study revealed that Wnt5a expression is significantly upregulated in the lesional skin of patients with psoriasis (12). Therefore, Wnt5a may contribute to the abnormal proliferation, differentiation and inflammatory response of keratinocytes in psoriasis. The present study aimed to determine the molecular mechanisms underlying the effect of Wnt5a in human epithelial cells and to investigate the role of Wnt5a in psoriasis.

Materials and methods

Cell culture. HaCaT cells were purchased from China Infrastructure of Cell Line Resources (Beijing, China). All cells were cultured in minimum essential medium/Earle's
balanced salt solution (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with foetal bovine serum (FBS; Corning Life Sciences, New York, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone, GE Healthcare Life Sciences). All cells were maintained at 37˚C in a humidified incubator with 5% CO₂. HaCaT cells in logarithmic growth phase were treated with either 40 or 80 ng/ml recombinant human Wnt5a (R&D Systems, Inc., Minneapolis, MN, USA).

Cell viability assay. HaCaT cells were seeded into 96-well plates at a density of 1x10⁴ cells/ml and incubated in medium containing 0.1% FBS overnight prior to treatment with either 40 or 80 ng/ml recombinant human Wnt5a for 24 h. The viability of HaCaT cells was investigated using Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Survival rates were calculated according to the following formula: Cell survival rate (%) = [optical density (OD) of treatment group - OD of blank group] / [OD of control group - OD of blank group] x 100. All experiments were repeated four times.

Cell cycle analysis using flow cytometry. The cell cycle of HaCaT cells was investigated using flow cytometry as previously described (12). HaCaT cells were seeded into 6-well plates at a density of 1x10⁵ cells/ml. The cells were treated with different concentrations of 0, 40 and 80 ng/ml Wnt5a for 24 h. The cells were then collected, washed twice with PBS, fixed with 70% ethanol overnight at 4˚C, treated with 1% RNase A for 30 min at 37˚C, and then stained with propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min at 4˚C. A total of 20,000 cells were counted per sample, and the cell cycle was analysed using an Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA). The cell cycle profiles were analyzed using the ModFit program version 3.1 (Verity Software House, Inc., Topsham, ME, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from different groups of cultured HaCaT cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized from 0.5 µg of total RNA using the GoScript™ Reverse Transcription System (Thermo Fisher Scientific, Inc., USA). The RT reaction was performed at 42˚C for 60 min and then at 70˚C for 5 min. The qPCR reaction was performed according to the protocol of the FastStart Universal SYBR Green Master (Roche Applied Science, Penzberg, Germany) using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., USA). The amplification program for all primers was 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The results were then analysed using the 2⁻ΔΔCt method (13), and determined values were then normalized to GAPDH expression as an internal control. Primers are listed in Table I.

Enzyme-linked immunosorbent assay (ELISA). The concentration of IL-8, IL-17A and IFN-γ in conditioned medium from cultured cells was detected using ELISA kits (EHC008, EHC170 and EHC102; Xin Bo Sheng Biological Technology Co., Ltd., Shenzhen, China). ELISAs were performed according to the manufacturer's protocol. The concentrations of IL-8, IL-17A and IFN-γ were calculated via comparison of the relative absorbance of the samples with the standards.

Statistical analysis. Statistical analyses were performed using SPSS version 21.0 (IBM Corporation, Armonk, NY, USA). The data are presented as the mean ± standard error of mean. One-way analysis of variance with a least square difference (LSD) was used to determine the statistical significance of results. P<0.05 was considered to indicate a statistically significant difference.

Results

Wnt5a reduces keratinocyte viability. The proliferative inhibitory effect of Wnt5a on HaCaT cell viability was investigated using the CCK8 assay. No significant difference in cell viability between the 40 ng/ml Wnt5a and the control group was observed (Fig. 1); thus, no cytotoxicity was considered to be present at this concentration. However, a higher concentration of Wnt5a (80 ng/ml) was significantly toxic to the cells (Fig. 1). Therefore, Wnt5a effectively inhibited the proliferation of HaCaT cells at the concentration of 80 ng/ml.

Effect of Wnt5a on keratinocyte cell cycle. Flow cytometry was used in order to investigate the effect of Wnt5a on HaCaT cell cycle progression (Fig. 2). Compared with the control group, Wnt5a significantly decreased the proportions of cells in the G0/G1 phase at both 40 and 80 ng/ml concentrations, whereas it significantly increased the proportion of cells in the G2/M phase at 80 ng/ml. These results suggest that Wnt5a arrested the keratinocyte cell cycle at the G2/M phase.

Wnt5a negatively regulates the expression of keratinocyte differentiation markers. In order to investigate the effect of Wnt5a on keratinocyte differentiation, the expression levels of filaggrin, keratin 1 and keratin 10 were investigated. The mRNA expression of these markers was determined using RT-qPCR (Fig. 3). The results demonstrated significant differences in the expression levels of filaggrin, keratin 1 and keratin 10 mRNA expression between the Wnt5a treatment groups (both 40 and 80 ng/ml) and the control group, thus suggesting that Wnt5a inhibited keratinocyte differentiation.

Wnt5a increases the expression of inflammatory-associated proteins in keratinocytes. The involvement of Wnt5a in the regulation of the inflammatory response in keratinocytes was investigated. ELISA was used to detect the expression of IFN-γ, IL-8 and IL-17A proteins in conditioned medium from cultured cells. Wnt5a significantly attenuated IFN-γ expression at both 40 and 80 ng/ml concentrations (Fig. 4A). Expression levels of inflammatory factors IL-8 and IL-17A were significantly enhanced by Wnt5a administration at the concentration of 80 ng/ml (Fig. 4B and C). These findings suggest that Wnt5a is an upstream mediator of the inflammatory response in keratinocytes.
Wnt5a downregulates the expression of β-catenin in keratinocytes. Exposure to Wnt5a for 24 h resulted in the suppression of β-catenin expression, the expression levels of downstream genes in the canonical Wnt pathway, as well as proliferation markers, were also investigated (Fig. 5B-D). RT-qPCR demonstrated that treatment with Wnt5a significantly reduced the expression of cyclin D1, Ki-67 and proliferating cell nuclear antigen (PCNA), thus suggesting that Wnt5a antagonized the canonical Wnt signalling pathway in keratinocytes.

**Discussion**

Wnt5a, a member of the Wnt family, has been determined to have diverse biological functions in organ development, cellular functioning, inflammatory responses and innate immunity (14,15). It has previously been demonstrated that Wnt5a is associated with numerous diseases, including cancer, diabetes, metabolic disorders and inflammatory diseases, specifically sepsis, atherosclerosis (16), rheumatoid arthritis (17), psoriasis vulgaris (18). Zhang et al (12) revealed that Wnt5a is significantly upregulated in psoriatic lesions compared with healthy skin. In the present study, it was demonstrated that treatment with Wnt5a inhibits cell proliferation and promotes an

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tbody>
<tr>
<td>Filaggrin</td>
<td>TGAAGCCTATGACACACCCTGA</td>
<td>TCCCCTACCGCTTTTCTGTCTCTT</td>
</tr>
<tr>
<td>Keratin 1</td>
<td>GCCAGTCTCAGGCTGAGGTTGTTT</td>
<td>TCTTCCCGTGAAAGGCTGGGACAAAT</td>
</tr>
<tr>
<td>Keratin 10</td>
<td>GAGCAAGGGAACTGACTACAG</td>
<td>CTCGGTTTCAGCTGAAATCT</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>AACTACCTGGACCGCTTCTCTT</td>
<td>CCACCTGAGCTTTGTTCACCA</td>
</tr>
<tr>
<td>PCNA</td>
<td>GGGTGAACCTCCACAGTAT</td>
<td>TTTCTTGTGGTTGCTTTC</td>
</tr>
<tr>
<td>Ki-67</td>
<td>TGACAAGGCCCGACTGATGAGAA</td>
<td>CTTTGGCTGCTGATGAGCTGTT</td>
</tr>
<tr>
<td>β-catenin</td>
<td>AAAATGGCAGTGGCTTTAG</td>
<td>TTIGAGGCAGTCTGCTGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGGAGTCAACGGATTGTCGTAT</td>
<td>AGCCCTTCCATGGGAGAC</td>
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PCNA, proliferating cell nuclear antigen.
inflammatory response in HaCaT keratinocytes. Therefore, the present study suggests that Wnt5a may have an involvement in the abnormal cell differentiation and inflammatory responses of keratinocytes in patients with psoriasis.

Previous studies have demonstrated the association between Wnt5a and inflammatory diseases, including psoriasis vulgaris, atherosclerosis and rheumatoid arthritis, suggests that Wnt5a may be involved in the development and pathogenesis of inflammatory disease (19,20). In addition, Wnt5a has previously been revealed to induce inflammatory responses in a variety of cell types, such as macrophages, cultured endothelial cells and synovial fibroblasts (20,21).
However, the role of Wnt5a in the inflammatory process associated with psoriasis remains unclear. In the present study, HaCaT cells that were treated with Wnt5a, which imitated the inflammatory response observed in patients with psoriasis. Furthermore, it was also revealed that Wnt5a stimulated the production of IFN-γ, IL-8 and IL-17A inflammatory factors. The expression levels of IFN-γ and IL-8 have previously been demonstrated to be upregulated in psoriatic lesions and anti-IL-17 therapeutic agents have previously been used for the treatment of psoriasis (1, 6, 22). Therefore, the findings of the present study suggest that Wnt5a has a pro-inflammatory function in HaCaT cells.

Histologically, psoriatic lesions are characterized by epidermal hyperplasia and altered epidermal differentiation, which are associated with the downregulation of keratinocyte differentiation markers (6, 22, 23). Vitamin D derivatives have been the first-line treatment for psoriasis for several years (24). A previous study demonstrated that administration of vitamin D can inhibit cell proliferation and promote differentiation in keratinocytes (24). Previous studies have also revealed that several late differentiation markers in keratinocytes, such as filaggrin and loricrin, are downregulated in psoriatic lesions (22, 25, 26). Furthermore, altered keratinocyte cell differentiation can lead to skin-barrier impairment in lesional skin (27). Currently available treatments for psoriasis, including retinoid and vitamin D administration, primarily target keratinocyte differentiation. Thus, keratinocytes may be involved in psoriasis pathogenesis. In the present study, filaggrin, keratin 1 and keratin 10 expression levels were reduced following treatment with Wnt5a, which suggests a role for Wnt5a in the differentiation of keratinocytes.

It has been previously established that Wnt5a is involved in the upregulation and the downregulation of cell proliferation (28, 29). For example, Wnt5a can promote the proliferation of chronic lymphocytic leukaemia cells (28) and endothelial cells, whereas it suppresses the proliferation of prostate cancer cells (29), B lymphocytes and epithelial ovarian cancer cells (9). In the present study, it was demonstrated that Wnt5a suppressed the growth of keratinocytes and regulated cell cycle progression at the G2/M phase. The findings of the present study are in contrast to the findings of Zhang et al. (12), as they revealed that a Wnt5a knockdown using small interfering RNA suppressed cell proliferation, induced apoptosis, and arrested cell cycle progression at the G0/G1 phase in keratinocytes. Therefore, further studies regarding the role of Wnt5a in keratinocyte proliferation in patients with psoriasis are required in order to verify the results of the present study.

Due to the diversity of Wnt5a-binding receptors and target cells/tissues, Wnt5a may activate and inhibit the β-catenin signalling pathway (10, 11). Previous studies have demonstrated that Wnt5a activates the β-catenin signalling pathway in pancreatic cancer cells, osteoblast-lineage cells and dermal fibroblasts (11). However, a previous study also revealed that Wnt5a suppressed β-catenin signalling during hair follicle regeneration (30). In the present study, it was revealed that Wnt5a negatively regulated the expression of β-catenin in HaCaT cells. Furthermore, canonical Wnt signalling genes (β-catenin and cyclin D1) and proliferation markers (Ki-67 and PCNA), were downregulated following treatment with Wnt5a. Cyclin D1 is an important regulator of cell cycle progression, and PCNA and Ki-67 are markers of cell proliferation (31, 32). These findings suggest

Figure 5. Effect of wingless-type family member 5a (Wnt5a) on the expression levels of downstream the canonical Wnt pathway genes and proliferation markers in HaCaT cells. Expression of (A) β-catenin, (B) cyclin D1 (C) PCNA and (D) Ki-67 in HaCaT cells at different concentrations of Wnt5a were investigated using reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. control, **P<0.01 vs. control. Wnt5A, Wnt family member 5A; PCNA, proliferating cell nuclear antigen.
that Wnt5a may inhibit the β-catenin signalling pathway in keratinocytes.

However, there were several limitations in the present study. Firstly, in order to determine the exact role of Wnt5a, experimentation using a wider range of Wnt5a concentrations would be required. In addition, the gene expression, protein level and cellular distribution of β-catenin were not investigated in the present study. With regards to psoriasis, the exact role of Wnt5a and its relationship with β-catenin signalling remain to be elucidated. In previous studies, increased nuclear β-catenin staining in the suprabasal epidermis in psoriatic lesions compared with normal skin has been identified (33). In contrast, Yamazaki et al (34) demonstrated that there was no β-catenin activation in psoriatic skin. Zhang et al (12); however, reported that Wnt5a is significantly upregulated in all of the epidermal layers in psoriasis lesions. Therefore, further investigation is required in order to determine the exact molecular mechanisms underlying the role of Wnt5a in psoriasis.

In conclusion, the present study has identified the roles of Wnt5a in keratinocyte responses that are relevant to psoriasis. In addition, the present study has demonstrated that exogenous Wnt5a may induce an inflammatory response, inhibit cell differentiation, downregulate β-catenin signalling and suppress the proliferation of keratinocytes. However, further studies are required in order to determine the in vivo function and mechanism of Wnt5a in the occurrence, development and relapse of psoriasis.

References