Tacrolimus reverses the pemphigus vulgaris serum-enhanced expression of desmoglein in HaCaT cells

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Abstract

Pemphigus vulgaris (PV) is associated with autoantibodies against desmoglein (Dsg), including Dsg1 and Dsg3. However, the precise mechanism by which acantholysis occurs in response to PV-IgG and the effect of tacrolimus for PV remain unclear.Method To co-culture human HaCaT keratinocytes with DMEM medium containing 5% PV-sera to establish a cell model of pemphigus that can determine the effect of PV-sera and tacrolimus on Dsg mRNA transcription and protein expression in HaCaT cells. Dsg protein expression in HaCaT cells was evaluated by Western blotting and Dsg mRNA transcription by real-time PCR (RT-PCR). The distribution of Dsg1 and Dsg3 in HaCaT cells was determined by indirect immunofluorescence (IIF).Results The application of 5% PV serum resulted in an increase in the transcription and expression levels of Dsg1 and Dsg3, whereas tacrolimus suppressed Dsg1 and Dsg3 expression. Tacrolimus inhibited PV serum-induced disruption of cell–cell contacts. Tacrolimus also downregulated the expression of Dsg1and Dsg3 compared with the PV group. IIF revealed that the linear deposits of Dsg1 on the surface of HaCaT cells in the PV-sera group disappeared and were replaced by granular and agglomerated fluorescent particles on the cell surface, whereas the Dsg3 linear deposits still existed, but this effect could be reversed by tacrolimus.Conclusion The Dsg3 antibody disrupts desmosome junctions by inducing endocytosis, resulting in desmosomal dissociation. Tacrolimus could reverse PV serum-induced enhancement Dsg expression in HaCaT cells.

Background

Pemphigus is a rare, chronic, potentially life-threatening, autoimmune blistering disease characterized by stained skin mucous crusting, erosion, and blisters in mucous membranes and skin. The aetiopathogenesis of pemphigus is characterized by acantholysis and intraepidermal blister formation, resulting from IgG autoantibodies directed against transmembrane desmosomal glycoproteins, including Dsg3 and/or Dsg1[1]. Based on clinical and pathological findings, pemphigus can be classified into four major forms: (i) pemphigus vulgaris (PV), (ii) pemphigus erythematosus, (iii) pemphigus foliaceus (PF), and (iv) proliferative pemphigus. Li et al. reported an increase in *Dsg3* mRNA abundance in HaCaT cells after culturing with the addition of sera of patients with PV and PF, whereas the fluorescence intensity of Dsg3 on the surface of HaCaT cells decreased[2]. However, their study neither detected Dsg3 expression in HaCaT cells nor employed a positive control.

In the traditional treatment of pemphigus, a combination of high-dose systemic corticosteroids with an adjuvant immunosuppressant is considered as main systemic treatment in pemphigus[1]. Tacrolimus (FK506) is calcineurin inhibitor that reduces T-cell activation, and topical application of tacrolimus has been studied in the treatment for mucosal and skin lesions in PV and PF, with beneficial results observed [3-5]. There are also two case reports on the successful use of systemic tacrolimus for recalcitrant PV unresponsive to prednisolone, azathioprine (AZA), mycophenolate mofetil (MMF), dapsone, and cyclophosphamide (CP) [6]. A randomized controlled trial demonstrated that tacrolimus effects are comparable to AZA as PV adjuvant treatment, although with less severe side effects [7]. Takae et al. used a pemphigus mouse model to evaluate various immunosuppressive therapies and reported the

suppressive effect of tacrolimus on the production of anti-Dsg3 IgG [8]. However, tacrolimus has not been studied at the cellular level, to date. This study aimed to explore the effect of PV-sera on mRNA and protein expression of Dsg1 and Dsg3 in HaCaT cells. The capacity of tacrolimus to reverse the effect of PV-induced Dsg upregulation in HaCaT cells was also assessed.

Results

5% PV-sera enhances *Dsg1* and *Dsg3* mRNA abundance in HaCaT cells and inhibited by tacrolimus

The mRNA transcription level of *Dsg1* and *Dsg3* in HaCaT cells incubated with 5% PV-sera is indirectly reflected by the amount of cDNA template detected by RT-PCR (Fig. 1a and b). Significantly higher *Dsg1* and *Dsg3* mRNA transcription levels were observed in the HaCaT cells in the presence of 5% PV-sera, whereas this effect decreased in the presence of 100 nM tarcrolimus (Fig. 1a for *Dsg1* and b for *Dsg3*, $P < 0.05$).

The addition of PV-sera increases Dsg1 and Dsg3 protein expression in HaCaT cells and is inhibited by tacrolimus

The expression of Dsg1 and Dsg3 in the PV-sera group significantly increased after the addition of PV-sera. However, when the cells were treated with PV-sera and tarcrolimus, the expression of Dsg1 and Dsg3 dramatically decreased (Fig. 1, c and d).

IIF analysis of Dsg1 and Dsg3 in HaCaT cells

We studied the pathogenic effect of PV-sera and the protective effect of tarcrolimus on HaCaT cells (Fig. 2). IIF revealed that incubation with 5% PV-sera induced specific changes in cell shape and formation of intercellular gaps. Under control conditions or following treatment with sera from healthy donors, Dsg1 and Dsg3 were continuously distributed along cellular junctions (Fig. 2a and c for Dsg1, and g and i for Dsg3). In contrast, 5% PV-sera treatment resulted in the disruption of Dsg1 and Dsg3 protein expression as indicated by weaker staining (Fig. 2b and h). Keratinocytes exposed to PV-sera showed that the linear Dsg1 distribution was absent while the linear Dsg3 distribution was remarkably reduced, which was similar with those incubated with Dsg3 monoclonal antibody (Fig. 2d and j). In the tacrolimus treatment group, incubation of HaCaT cells with PV-sera and tacrolimus strongly reduced shedding of Dsg3 from the cell surface (Fig. 2k), but did not reverse Dsg1 internalization (Fig. 2e).

Discussion

This study has shown that incubation with PV-sera can induce HaCaT cell colony contraction and formation of intercellular gaps, and gene transcription and protein translation levels of *Dsg1* and *Dsg3* in

HaCaT cells were significantly higher than that of the control. In addition, both PV-serum and anti-Dsg3 autoantibodies induced a disruption of the linear distribution of Dsg1, resulting in a dispersed distribution of smaller dots throughout the cytoplasm. In addition, our results confirmed that Dsg3 antibodies can result in Dsg1 antigen internalization on HaCaT cells. However, the linear distribution of Dsg3 was remarkably reduced in the HaCaT cells cultured with PV-sera compared to those of the control group and NH serum. The calcineurin inhibitor tacrolimus reversed the PV serum-induced transcription and expression of Dsg. The IIF results suggested that incubation of HaCaT cells with PV-sera and tacrolimus did not reverse Dsg1 internalization. The decrease in Dsg1 fluorescence may be related to protein endocytosis and its possible tendency of triggering a cascade reaction involving cytokines, and the expression of MMPs may lead to Dsg1/Dsg3 decomposition.

Studies demonstrating that the serum from pemphigus can affect the amount of Dsg in HaCaT cells are limited. Li Hui and her colleagues described that the serum containing anti-Dsg1 antibody decreased the expression of Dsg3 mRNA, and that containing anti-Dsg3 antibody increased the expression of Dsg3 mRNA, but Dgs3 protein expression decreased because of endocytosis of keratinocyte and the presence of anti-Dsg3 antibody in the serum[2] which was confirmed in this study using IIF. Lanza et al. reported that PV serum and PV IgG can induce acantholysis and reduce the total amount of Dsg3 in cultured keratinocytes, whereas linear epitopes of Dsg3 (anti-Dsg3-L IgG) fail to do so when administered at concentrations comparable to those present in pathogenic PV-sera[10]. However, the Dsg3-depleting activity of such polyclonal anti-Dsg3 IgG was acquired when the Dsg3 antibody was used at a concentration of 1 mg/mL, and this may be because of increasing of Dsg3 autoantibodies that bind to the epitopes at the surface of KC, which, in turn, hinders the binding of foreign antibodies, although autoantibodies preferentially bind to mature Dsg3-antigens and causes Dsg3 depletion from desmosomes. Dsg3 depletion may be due to the following: activation of p38MAPK, the binding of the autoantibodies leads to internalization of Dsg3, a collapse of the keratin cytoskeleton, and Dsg dissociation through MAPK pathway. Jolly et al. confirmed that PV IgG causes the internalization of cell-surface Dsg3 into endosomes (as early as 4 h), which are then depleted from both detergent-soluble and -insoluble pools, and this could be blocked by the p38MAPK inhibitor SB202190 [11]. The depletion of Dsg3 induced by autoantibodies from PV patients is dependent on PKC signaling [12]. Anti-Dsg3 antibodies have been shown to induce KC cell apoptosis, which may contribute to the depletion of Dsg3. Anti-Dsg3 antibodies increased the expression of mRNAs for proinflammatory cytokines (IL-1β, TNF-α, and IL-6), Bax, and uPAR, whereas decreased the levels of procaspase-3 and Bcl-2[13]. Dsg1-positive sera from patients can reduce the recognition of Dsg3 peptides. Anti-Dsg1 antibodies can recognize the epitopes of Dsg3 and thus anti-Dsg1 antibodies would bind to the peptides of Dsg3 and thus activated the p38MAPK/PKC pathway as well, which may result in the depletion of Dsg3[14, 15]. Serum containing anti-Dsg1 antibodies can decrease the expression of Dsg3 mRNAs, but serum containing anti-Dsg3 antibodies can increase the expression of Dsg3 mRNAs. The effect of Dsg1 on Dgs3 may be a negative feedback of KC, and anti-Dsg1 antibodies trigger the PKC pathway, which, in turn, causes a downregulation of Dgs3 mRNAs. Anti-Dsg1 antibodies can decrease the expression of Dsg3, while anti-Dsg3 antibodies can increase the expression of Dsg3. However, the increased expression of Dsg3 will be

consumed or offset through Dsg3 internalization as mediated by anti-Dsg3 antibodies or KC. Differences in the effects of anti-Dsg1and Dsg3 autoantibodies indicate that both Dsg1 and Dsg3 are not supposed to fully compensate for each other, and thus, are suggestive of the complexity of its pathogenesis.

Tacrolimus has been shown to potentiate the action of glucocorticoids by preventing their degradation, resulting in a steroid-sparing effect[16]. Interaction of desmoglein-3-reactive T cells with naïve B cells is required for induction of synthesis of pathogenic IgG[17], and thus, tacrolimus may be effective in controlling B-cell-mediated auto-immune in PV. In this study, an enhancement of the expression of Dsg1and Dsg3 by PV-sera was observed and reversed with the addition of 100 nM of tarcrolimus. Previous studies have been demonstrated that tarcrolimus inhibited the activation of p38MAPK pathway in atopic dermatitis, human colonic myofibroblasts and rheumatoid arthritis[18-20]. Thus, we assumed that tarcrolimus reserves the Dsg depletion by blocking the activation of p38MAPK pathway, which requires more in-depth research.

There is growing evidence suggesting that anti-Dsg autoantibodies cannot account for the loss of cell-cell adhesion as seen in PV, indicating that multiple combinations of pathogenic or subpathogenic autoantibodies may function together to contribute to acantholysis. “Desmoglein compensation hypothesis” and “steric hindrance theory” are the widely recognized pathogenic mechanisms of pemphigus[21-24]. The formation of blisters in PV is due to the synergistic effect of autoantibodies targeting multiple keratinocytes antigens [25, 26]. For example, in addition to Dsg, a variety of other autoantibodies have been described in PV, including cholinergic receptors [27], anti-mitochondrial Proteins [28], non-Dsg adhesion proteins (desmocollin and plakophillin) [29, 30], and additional targets concerning γδ-T lymphocytes, ATP2C1 and IL-36[31-33]. Fujimura et al. demonstrated that $CD163^{+}$ tissue-associated macrophages (TAMs) accumulate in the skin lesions of PV. The expression of periostin (POSTN), IL-36γ, and MMP-12 is prominent in the skin lesions of PV patients. In addition, serum levels of CXCL5 and sCD163 are significantly higher in PV patients compared to healthy donors [34]. These experiments provide key insights into the mechanism underlying multiple autoantibodies specificities that may be involved in blister formation in PV.

Conclusions

Taken together, our study has shown that PV serum can promote the transcription and expression of Dsg, and tacrolimus can reverse this effect of PV. Our findings may be utilized in elucidating the mechanism of action of tacrolimus in the treatment of pemphigus.

Methods

Participants and specimens

Serum samples were collected from seven patients with PV (Table 1) and three healthy volunteers. All the patients were in the active stage of the disease without treatment and met PV diagnostic criteria as

follows: (i) multiple flaccid, easily ruptured bullae on the basic of mucocutaneous erythema, (ii) progressive refractory erosions covered crust secondary to blisters, (iii) positive Nikolsky's sign, (iv) histopathological finding of intraepidermal blister formation, and (v) immunological feature of reticular bright green fluorescence IgG deposition on keratinocyte cells. We used the sera of healthy volunteers as control and inclusion criteria of PV as follows: (i) consistent with the diagnostic criteria for PV; (ii) neither immunosuppressant nor glucocorticosteroid was used in the last 30 days; (iii) without other autoimmune diseases, except for PV; and (iv) no significant organ dysfunction, as well as informed consent. Exclusion criteria are as follows: (i) PV patient is pregnant, (ii) pemphigus induced by drugs, (iii) patients with malignant tumor, and (iv) administration of tetracycline and macrolide antibiotics in the last month.

Cell culture and study design

The human keratinocyte cell line HaCaT (MssBio Co., Ltd. Guanzhou, China) was used to establish an *in vitro* model of PV according to a previous study[9].

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Cat. No. SH30022.01B) supplemented with penicillin (50 U/ mL, Hyclone, Cat. No. SH30010) with 10% fetal calf serum (Hyclone, Cat. No.SH30087.0), then seeded at a density of 2×10^5 cells/cm^2 on 25-mm^2 cell culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 24 h until the cultures reached 80% confluency. The HaCaT cells were subcultured in 12-well plates up to 80% confluency. The 3rd passages of HaCaT cells were cultured continuously for 24 h in DMEM high glucose medium containing 5% PV-sera at 37°C in a humid, 5% CO_2 incubator. For tacrolimus studies, cells were incubated in 5% PV-sera with 100 nM tacrolimus or NH sera with 100 nM tacrolimus (Absin Bioscience, China). The cells were harvested after 24 h, and total mRNA was extracted for analysis. Six experimental groups were designed in the study: control group (cells were incubated with medium without treatment), normal healthy (NH) sera group (cells were incubated with sera from healthy donors), PV-sera group (cells exposed to 5% PV-sera), positive group (cells were incubated with Dsg3 monoclonal antibody containing culture medium), PV-sera + FK506 group (cells were incubated with tacrolimus-treated and 5% PV-sera), and healthy-sera + FK506 group (cells were incubated with tacrolimus-treated and NH-sera).

Total RNA was extracted from the HaCaT cells using TRIzol reagent. First-strand cDNA synthesis was performed using a kit and following the manufacturer's instructions. Using NCBI for RT- PCR primer design, the primer sequences for amplification of the *Dsg1* (NCBI reference sequence: NM_001942), *Dsg3* (NM_001944), and *GAPDH* (NM_001289746) gene fragments are shown in Table 2. The reaction volume was 20 μL and prepared as follows: 5 μL of cDNA template (1:10 dilution, ViiA7 software), 0.5 μL of the upstream primer, 0.5 μL of the downstream primer, 10 μL of SYBR® Premix Ex Taq™ (Tli RNase H Plus) (2×), and 4.0 μL of ddH2O. PCR cycling conditions were as follows: incubation 95°C for 30s min, followed by 40 cycles of 95°C for 3 s and 60°C for 34 s, with the addition of 60°C 1 min for elongation.

Western blot analysis of Dsg expression

The HaCaT cells were grown in 12-well plates for 18 h, the medium was replaced with DMEM containing 5% PV-sera or NH sera, and incubated for another 18 h, then 1×10^8 to 1×10^9 cells were collected for western blot analysis. The primary antibodies used were mouse anti-Dsg3 antibody (1:160 dilution; Abcam, Cambridge, UK) and mouse anti-Dsg1 antibody (1:500 dilution; Abcam). Goat anti-mouse antibody (1:1,000 dilution, Alexa Fluor-488, Thermo Fisher Scientific, Dreieich, Germany) served as secondary antibody.

Indirect immunofluorescence (IIF) detection of Dsg in HaCaT cells

The cells were grown directly (1×10^5 cells/ml) on glass coverslips for 4 h in a 12-well plate and then continuously for 24 h after replacing the medium with or without supplements as earlier described. After washing with phosphate-buffered saline (PBS), the cells were fixed in 4% paraformaldehyde solution for 30 min. The samples were then washed thrice with PBS for 5 min each time. The cells were then permeabilized with 0.2% Triton X-100 for 5 min. After three rinses with PBS, the samples were blocked using 10% normal goat serum for 30 min. Then, the samples were incubated with the primary antibody (Dsg1 at 1:500 dilution; Dsg3 at 1:160 dilution) at 4°C overnight. As secondary antibody, fluorescently labeled goat anti-mouse antibodies were used at a dilution of 1:1,000 for 1 h at room temperature. The anti-Dsg1 and anti-Dsg3 antibodies were used against cell surface proteins Dsg1 and Dsg3. The nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). Images of the cells morphology were captured by standard light microscopy. An inverted fluorescence microscope (DMI6000B, Leica, Japan) was used for image acquisition at 40× magnification. Average OD value was assayed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA)

Statistical analysis

Western blot analysis was visualized using ImageJ (NIH, USA). Statistical significance was assessed using one-way ANOVA followed by Bonferroni correction using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) for comparison of multiple groups. Differences were deemed significant when the calculated p value was <0.05. The data were expressed as the mean ± SD.

Abbreviations

PV: Pemphigus vulgaris; Dsg: desmoglein; azathioprine: AZA; mycophenolate mofetil: MMF; CP: cyclophosphamide; NH: normal healthy; PBS: phosphate-buffered saline; DAPI: 4',6-diamidine-2'-phenylindole dihydrochloride

Declarations

Ethics approval and consent to participate

This study and all relevant experiments were reviewed and approved by Guangzhou Institute of Dermatology Research Ethics Committee (NO.201802). The purpose of the study, type and amount of specimen needed were explained to the participants and written informed consent was obtained from all study participants recruited in this study. The methods were conducted in accordance with approved guidelines and regulations.

Consent for publication

Not Applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

ZMX and QLP reviewed the literature and drafted the manuscript. XDY conceptualized the research design and revised the draft, XCS and YZ handled samples, and XND analyzed the data. All authors were involved in revising the manuscript and approving the final version.

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Tables

Table 1 Description of seven PV patients in the study

Case	Gender	Age	Brief description of case history
1	F	44	Progressive erythema, erosion with filthy crust in trunk for 2 weeks
2	M	43	Erythema, blister on extremities and trunk for 3 months
3	F	32	Recurrent painful erythema and blisters all over the body for half a year
4	F	40	Erythema, blisters, erosion with pain all over the body for more than 2 months
5	M	63	Oral ulcers for 3 months, recurrent blisters on extremities and trunk for more than 1 month
6	M	72	Trunk and oral blisters, erosions with pain for 2 months
7	F	56	Erythema, blisters, erosions with pain all over the body for more than 3 months

Table 2 Description of primer sequences included in the study

Gene	Sequence	Locus at target gene	PCR product size
Dsg1	F 5'-GGCATTCAATCCGAAGGCAG-3'	263-282	112 bp
	R 5'-AGTGAATTTTGGCGATTGGGTT-3'	364-343	
Dsg3	F 5'-TTGAGCTGCTTGGAAAAAGGGA-3'	3,889-3,910	73 bp
	R 5'-TATATGGCTTCCCAGCACCAAG-3'	3,961-3,940	
GAPDH	F 5'-TGTTGCCATCAATGACCCCTT-3'	406-426	202 bp
	R 5'-CTCCACGACGTACTCAGCG-3'	607-589	

Figures

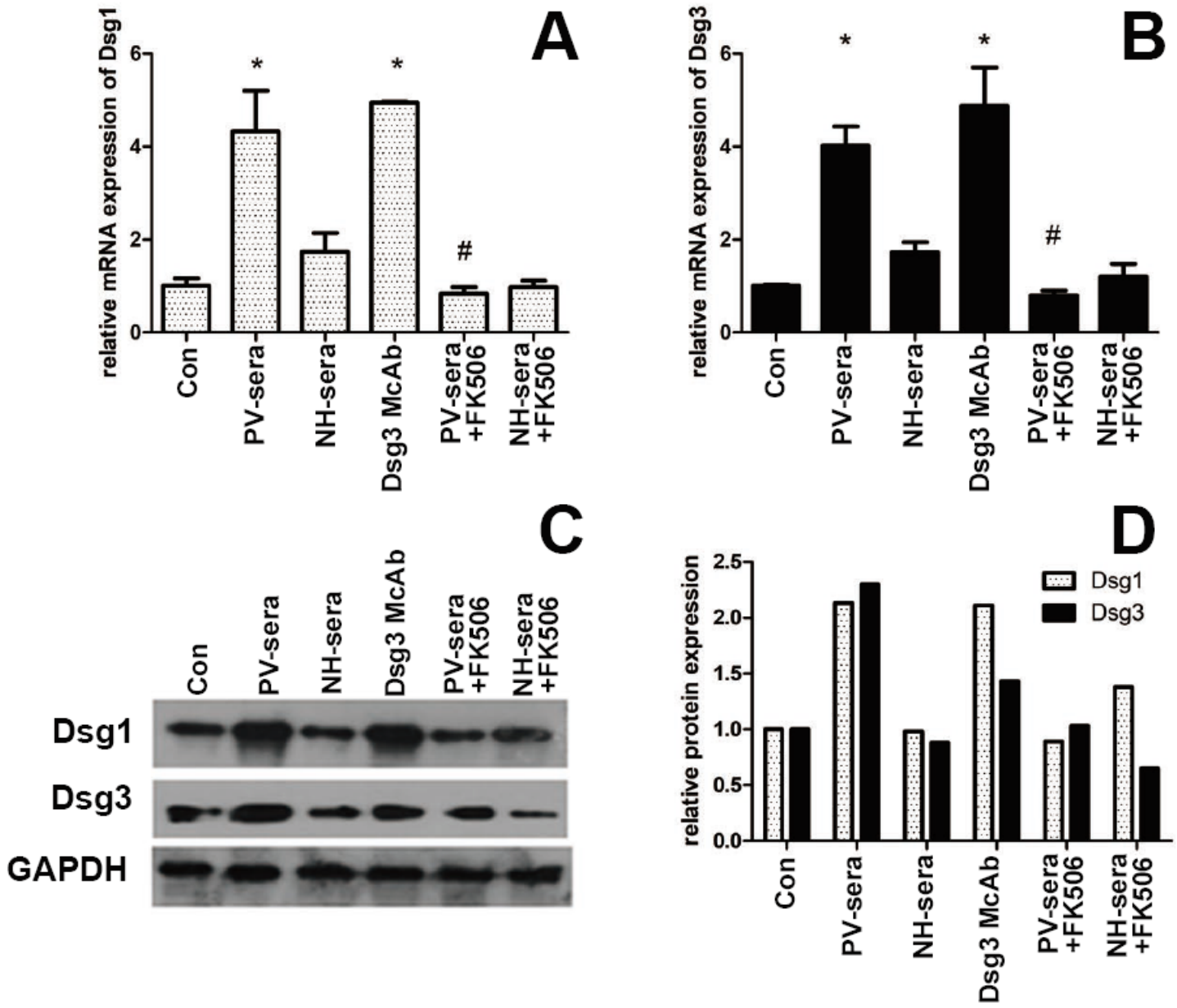

Figure 1

Dsg1/Dsg3 transcription and expression levels in HaCaT cells under different medium conditions. The effect of PV-sera on Dsg1and Dsg3 mRNA abundance of in HaCaT cells measured by RT-PCR (a, b), mRNA levels were normalized to that of GAPDH (=1). Each bar represents the mean ± SD. (*P <0.05 vs. control group, #P <0.05 vs. PV-sera group). One-way ANOVA was used to evaluate differences in the average Ct values among the four groups. The level of Dsg1 and Dsg3 expression was analyzed by western blotting (c), normalized using GAPDH, and plotted using GraphPad Prism (d). GAPDH was used as a control for SDS/PAGE loading.

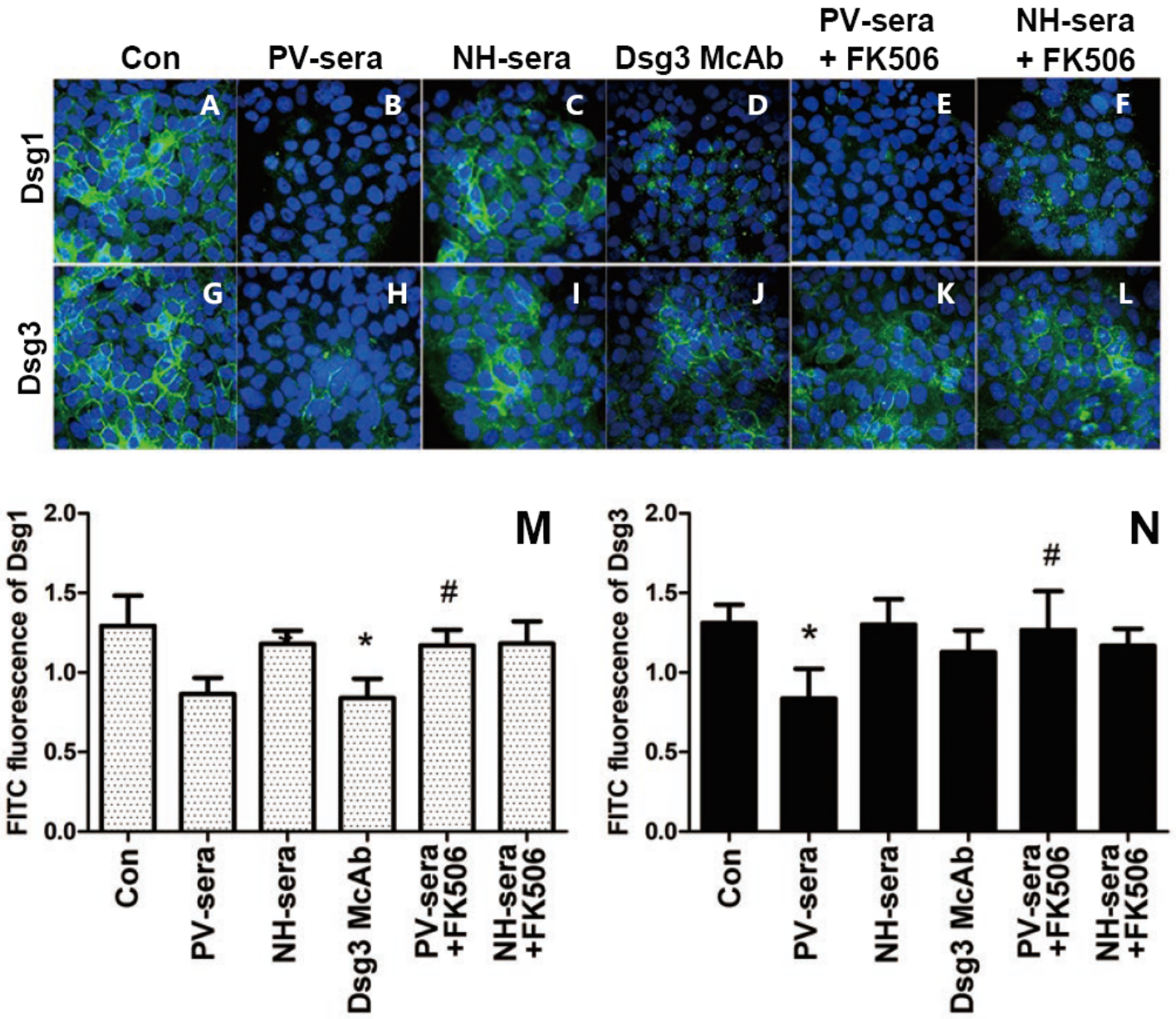

Figure 2

Immunofluorescence staining of anti-Dsg1/Dsg3 and histogram of optical density (OD) values at the surface of HaCaT cells as detected by IIF.Nuclear staining of HaCaT cells using DAPI. The cells were treated for 18 h by medium with supplements (a, g), 5% PV-sera (b, h), sera for normal healthy (NH) donors (c, i), Dsg monoclonal antibody (d, j), 5% PV-sera and tacrolimus (e, k), and NH-sera and tacrolimus (f, l). Dsg1 and Dsg3 levels were assessed by immunofluorescence, the graphics shows the median of FITC (green) fluorescence intensity (m, n). Each bar represents the mean ± SD (*P < 0.05 vs. control group, #P<0.05 vs. PV-sera group).