A Preliminary Study of the Effect of Semaphorin 3A and Acitretin on the Proliferation, Migration, and Apoptosis of HaCaT Cells

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Abstract
Background: Vascular endothelial growth factor (VEGF) is significantly elevated in psoriatic patients and is associated with the severity of the psoriasis. Due to the effect of inhibiting production of VEGF, acitretin can effectively treat psoriasis. Semaphorin 3A (Sema3A) restrains tumor growth and angiogenesis by partially reversing VEGF effects on tumor. However, the role of Sema3A in the pathogenesis of psoriasis is unclear.

Aims and Objectives: This study aimed to investigate the effect of VEGF, Sema3A, and acitretin on HaCaT cells, to see whether Sema3A could be a beneficial factor in psoriasis, as well as acitretin. Materials and Methods: Functional analysis of VEGF, Sema3A, and acitretin was carried out using HaCaT cells cultured under different treatments. Cell counting kit-8 method, colony formation assay, flow cytometry, transwell migration, reverse transcription-polymerase chain reaction, and Western blot test were performed to measure proliferation, colony formation, migration, apoptosis, and the expression of Bcl2, Bax, Caspase 3, and Caspase 9 of HaCaT cells.

Results: Sema3A and acitretin inhibited the proliferation, colony formation, and migration of HaCaT cells, while inducing the apoptosis of HaCaT cells by inhibiting the expression of Bcl2, and promoting the expression of Bax, Caspase 3, and Caspase 9, which were opposite to VEGF. Sema3A and acitretin partially reversed the function of VEGF.

Conclusions: Like acitretin, exogenous supplement of Sema3A may correct the abnormal proliferation and apoptosis procedure of HaCaT cells, and partially reverse the function of VEGF.

Key Words: Acitretin, HaCaT cells, psoriasis, semaphorin 3A, vascular endothelial growth factor

Introduction
Psoriasis is a chronic inflammatory skin disorder involving the interaction between immune cells and keratinocytes.¹,² Under the influence of activated T-cells and cytokines, psoriasis presents with hyperproliferation and abnormal apoptosis of keratinocytes in the epidermis³ and inflammatory infiltration and angiogenesis in the dermis.⁴ Activated keratinocytes could be a major source for vascular endothelial growth factor (VEGF) (also known as VEGFA). VEGF is overexpressed in serum and skin lesions of psoriatic patients and closely associated with clinical severity of psoriasis.⁵ It is proved that VEGF binds to VEGFRs resulting in excessive keratinocytes proliferation and migration, angiogenesis, and inflammatory infiltration which govern the pathological process in psoriasis, which is necessary for initiating, sustaining, and amplifying the pathophysiology of psoriasis.⁶ Experiments show the therapeutic effect of anti-VEGF therapy for the treatment of psoriasis is able to reverse a psoriasis-like skin phenotype. VEGF inhibitors, monoclonal antibody such as bevacizumab, and ramucirumab, used in the treatment of solid cancers are also effective for psoriasis.⁷

Acitretin, a second-generation synthetic retinoid which is approved for the systemic treatment of psoriasis and has been shown to improve the severity of psoriasis.⁸ Acitretin’s antipsoriatic effects are due to modifying gene transcription, promoting cellular differentiation, reducing keratinocyte proliferation, inducing apoptosis, and inhibiting inflammation. More important, acitretin inhibits keratinocyte production of VEGF and neutrophil chemotaxis.⁹

Semaphorin 3A (Sema3A) belongs to the semaphorins family which are originally known as evolutionary...
conserved axonal guidance molecules and closely related to tumor progression.\textsuperscript{[10]} Sema3A acts as a potent suppressor for tumor angiogenesis in various cancer models.\textsuperscript{[11]} Overexpression of Sema3A in oral cancer cells drastically suppresses tumor growth by inhibiting angiogenesis and tumor migration.\textsuperscript{[12]} However, the function of Sema3A in regulation of psoriasis has not been studied extensively and its effects in keratinocyte are not known.

In this study, we aimed to analyze the specific function of VEGF, Sema3A, and acitretin on the proliferation, colony formation, migration, and apoptosis of HaCaT cells, and the pathway they may affect on psoriasis.

\section*{Material and Methods}

\subsection*{Chemicals and reagents}

Recombinant human Sema3A protein (APL917Hu02), VEGF165 (100-20-2), and all-trans-acitretin (R106320) were purchased from Cloud‑Clone Technology Co. Ltd., (Wuhan, China), PeproTech (USA), and Aladding Co. Ltd., (Shanghai, China), respectively.

\subsection*{Cell culture and treatments}

Human keratinocytes (HaCaT cell lines) were obtained from the American Type Culture Collection, cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 lg/ml) (Invitrogen, Carlsbad, CA, USA), under a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. Active VEGF165, Sema3A, and acitretin were dissolved in dimethylsulfoxide and diluted with a culture medium. Cells were incubated with the indicated concentrations for the indicated time.

\subsection*{Cell counting kit-8 method}

Cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, cat\#CK04) was applied to incubate with HaCaT cells measured viability and proliferation, according to the manufacturer’s guidelines. The absorbance at 450 nm was read on a microplate reader (Molecular Devices, SpectraMax Plus 384). All experiments were performed in triplicate. Cell viability was calculated according to the following equation: Cell viability (%) = ([OD treatment–OD blank]/[OD control–OD blank]) × 100%.

\subsection*{Cell apoptosis detected by flow cytometry}

Cell apoptosis was detected by the Annexin V-FITC Apoptosis Detection Kit (GIBCO, cat\#V13242) according to the manufacturer's protocol. HaCaT cells were placed in six well plates with a density of $1 \times 10^5$/2 ml/well and cultured for 24 h. After treatment, cells were trypsin-released and Annexin-V/PI stained at room temperature and kept in dark place for 5 min. Apoptotic cells were measured by flow cytometer (BD Bioscience, Accuro C6) and quantitated.

\subsection*{Transwell assay}

HaCaT cells ($2 \times 10^5$ cells/2 ml/well) were seeded into six well plates for 24 h and different treatments were added for co-culture. Then, trypsin was released and cultured in 2% fetal bovine serum media. Cells of each group were placed within 24 well (5 $\times 10^4$ cells/0.5 ml/well) of transwell chambers with 8-mm pore size. Media that contained 10% fetal bovine serum was placed in the lower chambers as a chemoattractant. Cells were allowed to migrate for 16 h–24 h and then fixed with 4% paraformaldehyde for 20 min. At this point, they were stained with Crystal Violet (sigma, cat\#HT90132) for 10 min at room temperature. The number of migrated cells were counted and averaged randomly from five visual fields under a microscope. Migration was plotted as the percentage of control, as each cell line migrated differently.

\subsection*{Colony formation assay}

HaCaT cells were seeded as before. After full attachment, cells were trypsin-released and diluted to 100 cells/ml per well cultured in 10% fetal bovine serum, then incubated for 2–3 weeks (q96 h each group was exposed to correspondent treatments). After colonies formed (more than 50 cells), plates were washed with PBS and stained with Crystal Violet (sigma, cat\#HT90132) for 10 min and then photographed by ChemiDocTM MP System (Bio-Rad, USA).

\subsection*{Reverse transcription-polymerase chain reaction analysis}

Total RNA was obtained using 1 ml Trizol reagent (Baosai Biology #RE02050) according to the manufacturer’s instructions and stored at –80°C until use. Reverse transcription Kit (Baosai Biology #RT02020) was used for reverse transcriptase reactions. About 2 μg total RNA was reverse transcribed into cDNA in a reaction volume of 20 μL. Then, cDNA was polymerase chain reaction (PCR) amplified under fluorescent quantitative Kit (Baosai Biology #PM10003) instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Relative mRNA expression levels of all examined genes were measured using the comparative 2\textsuperscript{-ΔΔCt} All primers used are depicted in Table 1.

\subsection*{Western blotting}

The soluble protein extracts were separated by SDS-PAGE on a 10%–20% gradient gel and transferred to a Polyvinylidene difluoride (PVDF) membrane. Each blot membrane was incubated as per instruction with primary antibodies. The primary antibodies against Bcl2 (ab692), Bax (ab7977), Caspase 3 (ab32351), Caspase 9 (ab32539), and β-actin (ab63982) were purchased from Abcam. The blots were incubated with secondary goat anti-rabbit antibody (Abcam, ab63982) for 1 h at room temperature.
Protein bands were detected on X-ray film using an enhanced chemiluminescence detection system.

**Statistical analysis**

All data were presented as mean ± standard deviation of the mean and analyzed statistically using SPSS version 19.0 software (IBM SPSS Inc., Chicago, IL, USA). Statistical differences between data were evaluated by a two-tailed Student’s t-test. The comparison among three or more groups was performed by one-way analysis of variance. *P* < 0.05 was considered statistically significant.

**Results**

**Semaphorin 3A and acitretin inhibited the proliferation of HaCaT cells**

HaCaT cells were incubated with various concentrations of VEGF, Sema3A, and acitretin for 24–48 h. The CCK8 assay showed that VEGF promoted cell proliferation, Sema3A, and acitretin inhibited cell proliferation, in a dose-dependent manner, as shown in [Figure 1a-c]. VEGF at 4 ng/ml, Sema3A at 1 μg/ml, and acitretin at 10 μM were selected to incubate with HaCaT cells for 24 h. Sema3A and acitretin restrained cell proliferation significantly compared to the control group and compared to the VEGF group (*P*<0.001 in all cases). VEGF + Sema3A/VEGF + acitretin reduced cell proliferation significantly (*P*=0.004, *P*=0.003, respectively) compared to the VEGF group [Figure 1d]. It indicated that Sema3A and acitretin restrained cell proliferation, and partially reduced VEGF effect.

![Figure 1](image_url)

**Figure 1:** HaCaT cells were cultured in different groups: Vehicle control, vascular endothelial growth factor (vascular endothelial growth factor 1 ng/ml, 4 ng/ml, and 8 ng/ml), semaphorin 3A (semaphorin 3A 0.1 μg/ml, 0.5 μg/ml, and 1 μg/ml), vascular endothelial growth factor + semaphorin 3A (vascular endothelial growth factor 4 ng/ml + semaphorin 3A 1 μg/ml), acitretin (acitretin 0.1 μM, 1 μM, and 10 μM), vascular endothelial growth factor + acitretin (vascular endothelial growth factor 4 ng/ml + acitretin 10 μM) for 24 h. Cell viabilities of different groups were measured using the cell counting kit-8 assay. (a) Vascular endothelial growth factor promoted the proliferation of HaCaT cells in a dose-dependent manner and the optical concentration was 4 ng/ml. (b) Semaphorin 3A inhibited the proliferation of HaCaT cells in a dose-dependent manner, the optical concentration was 1 μg/ml. (c) Acitretin inhibited the proliferation of HaCaT cells in a dose-dependent manner and the optical concentration was 10 μM. (d) The optimal effect of each agent was vascular endothelial growth factor at 4 ng/ml, semaphorin 3A at 1 μg/ml and acitretin at 10 μM. Cell proliferation of different groups. *P* < 0.05, **P** < 0.005 versus the control group. *P* < 0.05, **P** < 0.005 versus vascular endothelial growth factor exposed groups.

### Table 1: Polymerase chain reaction primers used in this study

| Gene name | Primer sequences | Product length (bp) |
|-----------|------------------|---------------------|
| Human casp9-F | AAGGCAACAGGGTGGAAC | 142 |
| Human casp9-R | GCTTACGAAGGTTGCAACA | |
| H Bcl2-F4 | GGGAAACAGACACACAAACGAT | 80 |
| H Bcl2-R4 | TCTTATGTACCTTACAGCCCA | |
| bax-F | ATGGGCTTGACATTGGACCTT | 124 |
| bax-R | GCCAAAAAGATTGCTACAGGT | |
| Casp3-F | ATCAGCTGTTTTTGCTCAT | 92 |
| Casp3-R | TTCTGTGACCTTACGT | |
| vegf-F | GTTGTGTTGGTGGAGTGGTT | 83 |
| vegf-R | ATCTTGCTGTCCCTTCTGT | |
| GAPDH-F | GGTCGCTGAGCACAATTCGT | 135 |
| GAPDH-R | GTTCGCTGAGCACAATTCGT | |

**Figure 1:** HaCaT cells were cultured in different groups: Vehicle control, vascular endothelial growth factor (vascular endothelial growth factor 1 ng/ml, 4 ng/ml, and 8 ng/ml), semaphorin 3A (semaphorin 3A 0.1 μg/ml, 0.5 μg/ml, and 1 μg/ml), vascular endothelial growth factor + semaphorin 3A (vascular endothelial growth factor 4 ng/ml + semaphorin 3A 1 μg/ml), acitretin (acitretin 0.1 μM, 1 μM, and 10 μM), vascular endothelial growth factor + acitretin (vascular endothelial growth factor 4 ng/ml + acitretin 10 μM) for 24 h. Cell viabilities of different groups were measured using the cell counting kit-8 assay. (a) Vascular endothelial growth factor promoted the proliferation of HaCaT cells in a dose-dependent manner and the optical concentration was 4 ng/ml. (b) Semaphorin 3A inhibited the proliferation of HaCaT cells in a dose-dependent manner, the optical concentration was 1 μg/ml. (c) Acitretin inhibited the proliferation of HaCaT cells in a dose-dependent manner and the optical concentration was 10 μM. (d) The optimal effect of each agent was vascular endothelial growth factor at 4 ng/ml, semaphorin 3A at 1 μg/ml and acitretin at 10 μM. Cell proliferation of different groups. *P* < 0.05, **P** < 0.005 versus the control group. *P* < 0.05, **P** < 0.005 versus vascular endothelial growth factor exposed groups.
**Semaphorin 3A and acitretin-induced apoptosis of HaCaT cells**

The apoptosis of HaCaT cells was tested by flow cytometry. VEGF made no obvious difference on apoptosis of HaCaT cells. The Sema3A, VEGF + Sema3A, acitretin, and VEGF + acitretin groups induced cells apoptosis significantly ($P<0.001$, $P<0.001$, respectively) compared to the control group and ($P=0.001$, $P=0.001$, respectively) the VEGF group. Sema3A and acitretin were able to induce the apoptosis of HaCaT cells [Figure 2a and b].

**Semaphorin 3A and acitretin suppressed the migration and colony formation of HaCaT cells**

The transwell migration test was used to study the capacity of cell motility toward a chemoattractant gradient. Compared to the control group, VEGF significantly promoted the migration of HaCaT cells ($P=0.003$), while the migration were suppressed by Sema3A and acitretin ($P=0.023$, $P=0.019$, respectively). Compared to the VEGF group, the Sema3A, VEGF + Sema3A, acitretin and VEGF + acitretin groups decreased migration significantly ($P=0.003$, $P=0.003$, $P=0.002$, $P=0.006$, respectively) [Figure 3a and b].

![Figure 2: The apoptotic rate of HaCaT cells of different groups.](image-url)

(a) The apoptotic rate of the control group was 2.6%, vascular endothelial growth factor group was 3%, semaphorin 3A group was 22.4%, vascular endothelial growth factor + semaphorin 3A was 10.8%, acitretin group was 22.9%, and vascular endothelial growth factor + acitretin was 15.6%. (b) The control group and vascular endothelial growth factor group had no obvious change on apoptosis. Semaphorin 3A, vascular endothelial growth factor + semaphorin 3A, acitretin, and vascular endothelial growth factor + acitretin increased the apoptotic rate of HaCaT cells. *$P < 0.05$, **$P < 0.005$ versus control group. *$P < 0.05$, **$P < 0.005$ versus vascular endothelial growth factor exposed groups.
Colony formation assay showed consistent results and measured cell proliferation. Compared to the control group, VEGF promoted cell colony formation significantly ($P<0.001$). Compared to the VEGF group, the Sema3A, VEGF + Sema3A, and acitretin and VEGF + acitretin group decreased colony formation significantly ($P<0.001, P=0.001, P=0.002, \text{ and } P=0.001$, respectively) [Figure 4a and b]. It revealed that VEGF promoted cellular migration and colony formation, while Sema3A and acitretin had inhibited function and partially reduced VEGF effect.

The alternative mRNA expression of Bcl2, Bax, Caspase 3, and Caspase 9 affected by vascular endothelial growth factor, semaphorin 3A, and acitretin

Bcl2, Bax, Caspase 3, and Caspase 9 have been implicated in the pathogenesis of psoriasis. The mRNA expression was tested by reverse transcription-PCR (RT-PCR) after HaCaT cells harvested with VEGF, Sema3A, and acitretin for 24 h. The data revealed that VEGF elevated Bcl2 and VEGF expression significantly ($P=0.020, P=0.016$, respectively) and reduced Bax, Caspase 3, and Caspase 9 expression significantly ($P=0.033, P=0.036, P=0.002$, respectively) compared to the control group.

Compared to the control group and VEGF group, Sema3A decreased Bcl2 expression ($P=0.025$ and $P=0.003$, respectively), increased Bax ($P=0.003$ and $P<0.001$, respectively), Caspase 3 ($P=0.004$ and $P=0.001$, respectively), and Caspase 9 expression ($P=0.001$ and $P=0.001$, respectively). The VEGF + Sema3A group decreased Bcl2 and VEGF expression ($P=0.001, P=0.04$, respectively), and increased Caspase 9 expression ($P=0.031$) compared to the VEGF group, and had no statistically significant effect on Bax and Caspase 3 expression compared to the VEGF group.

A similar trend was shown in the acitretin group. When compared to the VEGF group, acitretin...
decreased Bcl2 and VEGF expression ($P = 0.001$, $P = 0.03$, respectively). Compared to the control group and the VEGF group, acitretin increased Bax ($P = 0.003$, $P = 0.005$, respectively), Caspase 3 ($P = 0.028$, $P = 0.022$, respectively), and Caspase 9 ($P = 0.004$, $P = 0.002$, respectively) expression. The VEGF + acitretin group elevated Bax, Caspase 3, Caspase 9 expression significantly ($P = 0.02$, $P = 0.002$, $P = 0.03$, respectively), decreased Bcl2 ($P = 0.001$) and VEGF ($P = 0.02$) expression compared to the VEGF group.

It indicated that Sema3A and acitretin reduced the expression of Bcl2 and enhanced the expression of Bax, Caspase 3, and Caspase 9, partially reduced VEGF effect [Figure 5].

The alternative protein expression of Bcl2, Bax, Caspase 3, and Caspase 9 affected by vascular endothelial growth factor, semaphorin 3A, and acitretin

The protein expression of Bcl2, Bax, Caspase 3, and Caspase 9 was analyzed by Western blot after HaCaT cells
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processed as before. Downregulation of Bax, Caspase 3, and Caspase 9 were observed in the VEGF group. The expression of BclL decreased and Bax, Caspase 3, and Caspase 9 increased in the Sema3A and acitretin group. In the VEGF + Sema3A group and VEGF + acitretin group, BclL decreased, Caspase 3, and Bax, Caspase 9 increased compared to the VEGF group. It indicated that Sema3A and acitretin influenced over apoptosis by reducing Bcl2 expression and enhancing Bax, Caspase 3, and Caspase 9 expression [Figure 6].

Discussion

VEGF is considered as one of the most potent angiogenic factors that govern angiogenesis and affect keratinocytes in psoriasis.[13,14] Our experiment indicated that HaCaT cells showed excessive proliferation, invasiveness, and migration due to VEGF stimulation, which was consistent with the current research.[15] The inhibition of VEGF may effectively improve psoriasis.[7] In our experiments, the exogenous supplementation of Sema3A and acitretin was able to inhibit the proliferation, migration, and colony formation of HaCaT cells. More important, Sema3A and acitretin partially reduced the effect of VEGF. The resistance to apoptosis is one of the important factors implicated in the pathogenesis of psoriasis, leading to an increased number of keratinocytes which undergo proliferation.[16] The regulation of apoptotic processes in psoriatic keratinocytes involves a dynamic interaction between the Bcl gene family in which different members exert the opposite function on the regulation of apoptosis.[17,18] Bcl2 gene can prevent cell from apoptosis, while Bax gene can induce cell apoptosis.[19] The expression of Bcl2 and Bax in psoriatic keratinocytes is controversial; some research supported the theory that increased Bcl2 expression and decreased Bax expression coexist in psoriatic lesions leading to keratinocytes resistance of apoptosis. During the early stages of apoptosis, activated Caspase 3 is regarded as a marker of an apoptosis execution entry point. Caspase 3 and Caspase 9 levels are downregulated in psoriatic lesion skin biopsies compared to nonlesion skin biopsies, which decrease apoptotic rate in psoriasis.[20,21]

We found that Sema3A and acitretin were able to induce HaCaT cell apoptosis, which was contrary to the function of VEGF. We then investigated the effect of VEGF, Sema3A, and acitretin on the mRNA and protein expression of Bcl2. Bax, Caspase 3, and Caspase 9, respectively, to see the pathway that they may work on. The results suggested that VEGF influenced over the apoptosis of keratinocyte by activating Bcl2 and suppressing Bax, Caspase 3, and Caspase 9. Sema3A and acitretin reduced Bcl2 expression and promoted Bax, Caspase 3, and Caspase 9 expression to induce the apoptosis of keratinocytes. Then, we proceeded to coculture HaCaT cells overexpressing VEGF and Sema3A, VEGF and acitretin to see their interaction. Even though, the mRNA expression of Bcl2, Bax, Caspase 3, and Caspase 9 had delicate differences in protein expression, they all demonstrated that Sema3A and acitretin could partially reversed the function of VEGF to reduce Bcl-2 expression and promote Bax, Caspase 3, and Caspase 9 expression in vitro.

Sema3A belongs to the class 3 semaphorins family which is originally described as a secretory protein with potent axonal repulsive activity.[22] It is reported that overexpression of Sema3A significantly suppresses in vivo breast tumor growth in mice xenograft models and decreased expression is usually associated with poor prognosis in various cancers.[23] Sema3A can partially reverse the effects caused by VEGF in hematopoietic and leukemic cells. Sema3A compete with VEGF for binding their co-receptors Nrp-1/Nrp-2 to inhibit mitogenic effects of VEGF in endothelial cells, leading to apoptosis and inhibition of migration in breast cancer cell lines.[24] Sema3A may act as a potent tumor suppressor in tumor cells growth by inhibiting tumor angiogenesis and VEGF function.[25-27] Little is yet known about the role of Sema3A in the pathogenesis of psoriasis. Activated keratinocytes in psoriasis have the feature of excessive growth, which is similar to what is seen in tumor cells, but in benign progression. Keratinocytes in the epidermis express Sema3A and VEGF co-receptors Nrp-1 which has been found to mediate keratinocyte migration. We hypothesized that Sema3A may play a significant role in psoriasis through effecting keratinocyte and VEGF, as it does in tumor cells, and Sema3A may have a therapeutic effect in psoriasis as acitretin which has been proven useful in the treatment of psoriasis.
Our study found that the effect of Sema3A was consistent with acitretin. Sema3A and acitretin inhibited proliferation, migration, colony formation of keratinocytes in vitro, and promoted apoptosis through influencing over Bcl2, Bax, Caspase 3, and Caspase 9 expression. Both of them were able to partially reverse the function of VEGF on keratinocytes. These data partly strengthen our hypothesis that Sema3A might have the function to regulate aberrant biological characteristics of keratinocytes and reverse the function of VEGF. It is reasonable to believe that Sema3A might play a beneficial role in the pathogenesis of psoriasis and exogenous supplementation could be a prospective treatment for psoriasis in the future, just like acitretin. Based on the effect of Sema3A on keratinocyte in vivo, further study about the effect of Sema3A on psoriatic animal model in vitro is necessary to be carried out, we are looking forward to finding out more potential function of Sema3A in psoriasis.

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Conflicts of interest
There are no conflicts of interest.

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