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Original Article

The effect of calcitonin gene-related peptide on T lymphocyte infiltration and its role in the therapeutic effects of acitretin in psoriasis vulgaris

Dai Li[1,2], Ming-Liang Chen[1], Ming-Zhu Yao[1], Yue-Han Wu[3], Shuang Zhao[1], Juan Su[1], Xiang Chen[1]

[1]Department of Dermatology, Xiangya Hospital, Central South University, Changsha, China; [2]National Institution of Drug Clinical Trial, Xiangya Hospital, Central South University, Changsha, China; [3]Department of Pharmacology, School of Pharmaceutical Science, Central South University, Changsha, China

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Abstract: Background: The pathogenesis of psoriatic plaque lesions is related to the overexpression of calcitonin gene-related peptide (CGRP). We investigated changes in circulating and skin CGRP content, and their impacts on T lymphocyte infiltration and acitretin therapy in psoriasis vulgaris. Methods: CGRP expression was examined through immunohistochemistry of skin biopsies, and its serum levels were determined by radioimmunity in both patients with psoriasis vulgaris and healthy volunteers. Correlation between serum CGRP levels and Psoriasis Area and Severity Index (PASI) scores was analyzed. Effects of CGRP on adhesion, migration, and IL-6 secretion of T lymphocytes of healthy subjects were assessed. After psoriasis patients were treated with acitretin, CGRP I and II mRNA expression of T lymphocytes was determined by real-time polymerase chain reaction, and the CGRP contents of serum were assayed (via radioimmunity). T lymphocytes from healthy volunteers were obtained to detect the effect of acitretin on CGRP secretion. Results: CGRP expression levels in the skin and serum were higher in psoriasis patients than in healthy subjects. CGRP levels in psoriasis patients were correlated with their PASI score. CGRP facilitated T lymphocyte adhesion, migration, and IL-6 secretion. Treatment with acitretin inhibited CGRP I and II mRNA expression in T lymphocytes and serum CGRP levels in patients with psoriasis vulgaris. Acitretin inhibited CGRP secretion in T lymphocytes of healthy volunteers. Conclusions: CGRP is involved in derma T lymphocyte infiltration of psoriasis vulgaris, and the therapeutic effects of acitretin are related to decreases in CGRP synthesis and the release of circulatory T lymphocytes.

Keywords: Calcitonin gene related protein (CGRP), psoriasis, T lymphocytes, acitretin

Introduction

Psoriasis is a common, complex disease and its pathogenesis is not fully understood. Although the pathological role of the immune system and/or epidermal tissue of the skin in psoriasis has been exhaustively explored [1, 2] the role of the cutaneous neural system remains unclear. Many studies suggest that the nervous system may play a role in psoriasis, including in peripheral sensory-nerve interactions with immunomodulatory networks and keratinocyte proliferation, which promotes sensory nerve transmitters [3, 4]. Calcitonin gene-related peptide (CGRP) is a primary transmitter in the peripheral sensory nerve and its excessive release has been detected in psoriatic skin [5]. Our own study has shown that the CGRP level of plasma is increased in psoriasis patients compared to healthy subjects [6]. However, the detailed mechanisms of CGRP, inducing psoriasiform symptoms, are not clear.

Psoriasis is a chronic inflammatory dermatosis dependent on a variety of T cells, including $CD4^+$ helper T cells and $CD8^+$ T cells [7, 8]. T lymphocytes can migrate and adhere to skin lesions in the presence of chemotactic and/or proinflammatory substances in psoriasis [9]. Thus, the capacity to infiltrate is fundamental to the ability of T lymphocytes to cause psoriasiform dermatoses.

Acitretin is a second-generation retinoid. It is an alternative treatment for psoriasis [10]. The mechanism of the efficacy of the drug is not

fully understood. Its use results in a wide range of actions in various cellular types, such as immune cells [11]. Interestingly, retinoids (etinoic acid or retinoic acid) may themselves separately inhibit CGRP levels in different types of cell, such as rat carcinoma cells from the CA-77 C line or F9 cells [12, 13].

The discovery of the morbigenous and therapeutic roles of circulating CGRP in psoriasis vulgaris was significant. In the present study, we attempted to identify the role of CGRP in psoriasis vulgaris and the treatment mechanism of acitretin on psoriasis. Furthermore, the effects of CGRP on T lymphocytes were also observed.

Methods and materials

In vivo experiments

Subjects: The present study involved 62 patients diagnosed with active psoriasis vulgaris on the basis of both clinical features and a skin biopsy. Patients were included if they were aged between 14 and 58 years, had no significant infections or immunosuppression, and were free from significant renal, hepatic, or other type of disease.

Patients were excluded if they had received anti-psoriasis drugs within the previous month or any biological therapies within the previous 6 months. Some patients had only been given 0.5 mg/kg/day acitretin within 1 month.

We assessed the clinical severity of psoriasis using the Psoriasis Area and Severity Index (PASI) [14]. In the acitretin-treated group, 12 patients were only given 0.5 mg/kg/day acitretin within 1 month. As a control group, 60 age-matched healthy volunteers were also enrolled.

The study protocol was approved by the Ethics Committee of our Hospital. Written informed consent was obtained from patients after the nature, purpose, and potential risks of the study had been explained to them. The study was carried out in accordance with the Declaration of Helsinki.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were prepared from skin specimens of 12 psoriasis patients (randomly selected from the 62 patients) and 12 controls (obtained from normal tissues of patients who had undergone skin biopsy due to skin diseases such as psoriasis, cutaneous tumor, and so forth) and mounted on 3-aminopropyltriethoxysilane-coated slides, which were then processed for immunostaining using a two-step immunoperoxidase technique [15]. The tissue sections were dewaxed and hydrated in gradient alcohol. After microwave antigen retrieval, 0.3% H_2O_2 was used to block endogenous peroxidase activity in incubation with tissue sections for 30 min. The sections were further incubated for 20 min with blocking serum (Zhongshan Golden Bridge Biological Technology Co., Beijing, China) followed by incubation with commercially available rat monoclonal antibody against human CGRP (Sigma, Saint Louis, Missouri, USA) (1:150) at 4°C overnight. Sections that were not incubated with the primary antibody were used as negative controls. The slides were washed in phosphate buffered saline (PBS) and 0.1% Triton X-100, then incubated for 30 min with peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biological Technology Co., Beijing, China) at room temperature. The sections were developed in diaminobenzidine and then counter-stained with hematoxylin. All evaluations were carried out using a microscope (OLYMPUS CX41, Japan) by three independent observers blinded to patient data. Each digital image was separately and uniformly thresholded for the density slicing of the staining. Distinct epidermal compartments were then individually analyzed to compute the area fraction of positive staining.

Isolation of peripheral $CD3^+$ lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep (Sigma, Saint Louis, Missouri, USA) from 10 mL blood collected into a tube containing ethylenediaminetetraacetic acid (EDTA). PBMC subpopulations $CD3^+$ were isolated by positive selection using a magnetic cell separation system with human CD3 MicroBeads (MACS, Miltenyi Biotec GmbH; Bergisch-Gladbach, Germany).

The purity of each population was verified by flow cytometry (Canto II, BD, San Jose, USA) using the following antibodies: PE mouse anti-human CD3 (BD Pharmingen™, San Jose, USA). The purity of the separated $CD3^+$ was no lower than 91.2%. Purified lymphocytes were resus-

pended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, California, USA) with 1 μ/mL phytohaemagglutinin (PHA) at a density of 1.5×10^6 cells/mL. Then 10% heat-inactivated fetal calf serum was added to each flask, and cells were kept in culture for 24 h (atmosphere of 5% CO_2, 37°C). Cell viability was monitored by trypan blue dye to ensure a rate higher than 90% in all cases. $CD3^+$ lymphocytes were prepared for subsequent *in vitro* experiments, including adhesion assay, migration assay, IL-6 release assay, and real-time polymerase chain reaction (RT-PCR) analysis.

Real-time polymerase chain reaction analysis

Total RNA was extracted from T lymphocytes using a Trizol reagent. From each sample, 1 g RNA was reverse-transcribed. Quantitative analysis of CGRP mRNA expression was performed using the SYBR Green method on an ABI 7300 RT-PCR system (ABI, Carlsbad, California, USA) as in our previous report [16]. Sequences of primers were as follows: CGRP I: 5-CCCAGAAGAGAGCCTGTGACA-3 and 5-CTTCACCACACCCCCTGATC-3; CGRP II: 5-TCTTTCGGAGCCATCCTGTT-3 and 5'-GATTTCACGTCCCCCTAAGGTT-3' glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5-CTGCACCACCAACTGCTTAG-3 and 5-AGGTCCACCACTGACACGTT-3 [17] (Sangon, Shanghai China). Results were expressed as the ratio of CGRP I, II mRNA to GAPDH mRNA.

Measurement of CGRP content in plasma

For the measurement of plasma CGRP concentration, blood samples (2 mL) were collected in tubes containing 10% Na_2EDTA (30 μL) and aprotinin 400 U/mL. Plasma was obtained by centrifugation (1000 g, 15 min, 4°C), and then frozen at -20°C until assays were performed. To determine the immunoreactivity of CGRP in the plasma, a commercially available rabbit anti-human CGRP radioimmunoassay kit (Dong-Ya, Beijing, China) was used. This antibody has 100% reactivity with human CGRP I and 79% with CGRP II. There is no cross-reactivity with human amylin, calcitonin, somatostatin, or substance P.

In vitro experiments

Protocols: Our *in vitro* experiments were designed to seek direct evidence that endogenous CGRP has effects on adhesion, migration, and IL-6 release. T lymphocytes from healthy subjects were prepared for the following three series of experiments *in vitro*.

The first series was designed to evaluate the effects of exogenous CGRP on cell adhesion. Experiments were divided randomly into four groups, as follows: (1) a control group, T lymphocytes treated with PBS; (2) a low-dose CGRP (Sigma, Saint Louis, Missouri, USA) group, T lymphocytes treated with CGRP (5×10^{-8} mol/L) for 2 h; (3) a high-dose CGRP group, T lymphocytes treated with CGRP (5×10^{-7} mol/L) for 2 h; and (4) a high-dose CGRP plus CGRP (8-37) (Sigma, Saint Louis, Missouri, USA) group: for this group, the procedure was the same as that of group (3) except for pretreatment with CGRP (8-37) (5×10^{-6} mol/L), a CGRP receptor antagonist, for 15 min.

The second series was designed to evaluate the effects of exogenous CGRP on T lymphocyte migration. The experimental design was the same as that of the first series.

The third series was designed to evaluate the effects of exogenous CGRP on T lymphocyte IL-6 release. The experimental design was the same as that of the first series.

The fourth series was designed to observe the effects of acitretin on CGRP secretion from T lymphocytes. Experiments were divided randomly into four groups, as follows: (1) control group, T lymphocytes treated with ethanol solution; (2) low-dose acitretin (Sigma, Saint Louis, Missouri, USA) group, T lymphocytes treated with acitretin (5×10^{-8} mol/L) for 48 h; (3) middle-dose acitretin group, T lymphocytes treated with acitretin (5×10^{-7} mol/L) for 48 h; and (4) high-dose acitretin group, T lymphocytes treated with acitretin (5×10^{-6} mol/L) for 48 h. Acitretin was dissolved in ethanol in all groups.

T lymphocyte adhesion assay: Microtiter 96-well plates (Costar, Cambridge, MA, USA) were coated overnight at 4°C with 50 mg/L 1:8 metrigel solution (BD, San Jose, USA), followed by blocking with 10 g/L bull serum albumin (BSA) for 30 min at 37°C. Then they were washed with a medium (RPMI 1640 without additives). Cells (1×10^5 cells/mL) were allowed to attach for 2 h at 37°C with or without CGRP, and then nonadherent cells were removed by gentle aspiration and rinsing with prewarmed

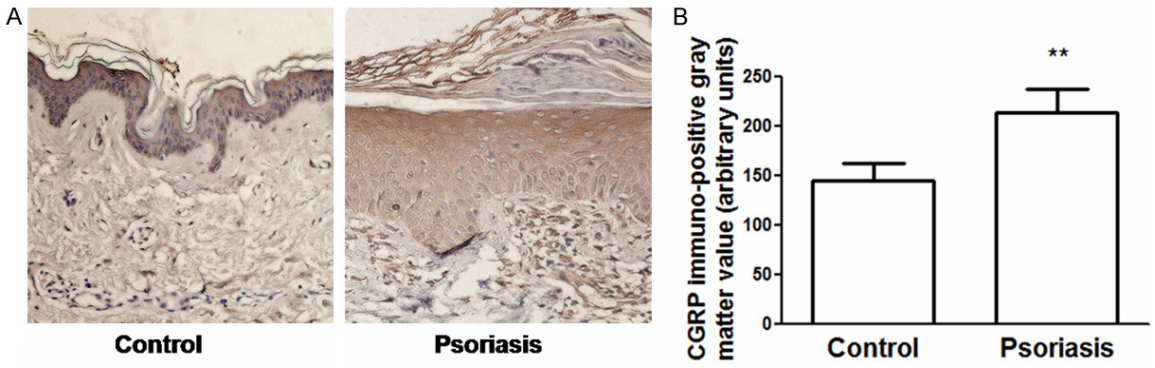

Figure 1. The expression of CGRP on skin of psoriasis patients is higher than on that of healthy subjects. Tissue sections were prepared from 12 patients with psoriasis and 12 healthy subjects and examined by immunohistochemistry. A: CGRP expression in normal skin tissue and in skin tissues of patients with psoriasis. Magnification, 200×. B: Quantification of CGRP expression in skin tissues of patients and healthy subjects. Results are shown as mean ± SE. $^{**}P<0.01$ vs. control.

RPMI 1640. Adherent cells were quantified using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method. Data are expressed as the mean of the percentage of adherent cells relative to total cell input, in three replicate wells.

T lymphocyte migration assay: Migration assays were performed using 24-well transwell plates with a 5.0 mm polycarbonate membrane (Corning Life Sciences, Corning, USA). Transwell plates were coated overnight at 4°C with 50 mg/L 1:8 metrigel solution (BD, San Jose, USA), and basement membrane hydration was performed prior to use. RPMI 1640 (600 μL) (GIBCO, USA) with different concentrations of CGRP or none at all was placed in the lower chamber. RPMI 1640 (100 μL), containing 5×10^4 cells, was placed in the upper chamber. Each experiment was performed in triplicate. After 24 h, migrated cells in the lower chamber were counted by 0.1% crystal violet staining. The number of migration cells was calculated by microscope in five random fields at 400×.

IL-6 release measurement: Cells were seeded at 1×10^4 cells/well in 24-well plates in the culture medium described above and incubated for 8 h. Then they were treated with CGRP (with or without CGRP (8-37) and incubated for a further 24 h. The IL-6 released from T lymphocytes was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA).

CGRP secretion assay: After cells were treated with different doses of acitretin, cell culture medium was collected. The amount of CGRP secreted from T cells was also measured by RIA, as in the previous description.

Statistical analysis

Data are expressed as mean ± SE and were analyzed using Statistical Product and Service Solutions (SPSS) 10.0 (IBM, Chicago, IL). Compared values were analyzed by Student's t test or one-way analysis of variance with the Student-Newman-Keulst test. $P<0.05$ was considered statistically significant. Spearman's correlation coefficient was applied to identify correlations between the PASI score and CGRP levels.

Results

CGRP expression is increased in skin and serum of psoriasis vulgaris patients

Immunohistochemical staining of skin tissue sections showed low levels of baseline expression of CGRP in healthy subjects (n=12) compared to psoriasis patients (n=12; **Figure 1A**). In psoriasis patients, CGRP expression was significantly increased at all epidermal layers (**Figure 1A**). There was a significant difference in mean expression score in the skin between psoriasis patients (212.94±23.81) and healthy subjects (144.62±17.65) (P=0.0072) (**Figure 1B**). These findings verified previous investigations showing that CGRP expression was significantly upregulated in patients with psoriasis vulgaris.

Serum levels of CGRP were significantly higher in psoriasis patients (43.58±4.79 pg/mL) th-

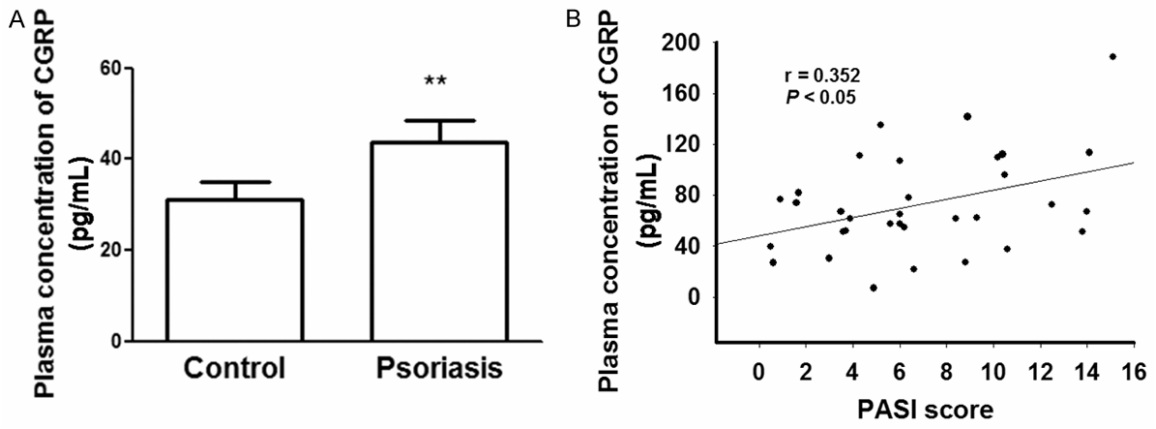

Figure 2. The relationship between plasma CGRP and psoriasis area and severity index. A: Serum CGRP levels in 32 patients with psoriasis vulgaris and 32 healthy subjects were determined by ELISA. Results are shown as mean±SE *P<0.05 vs. controls. B: CGRP levels positively correlated to PASI scores. r=0.352 *P<0.05 vs. controls.

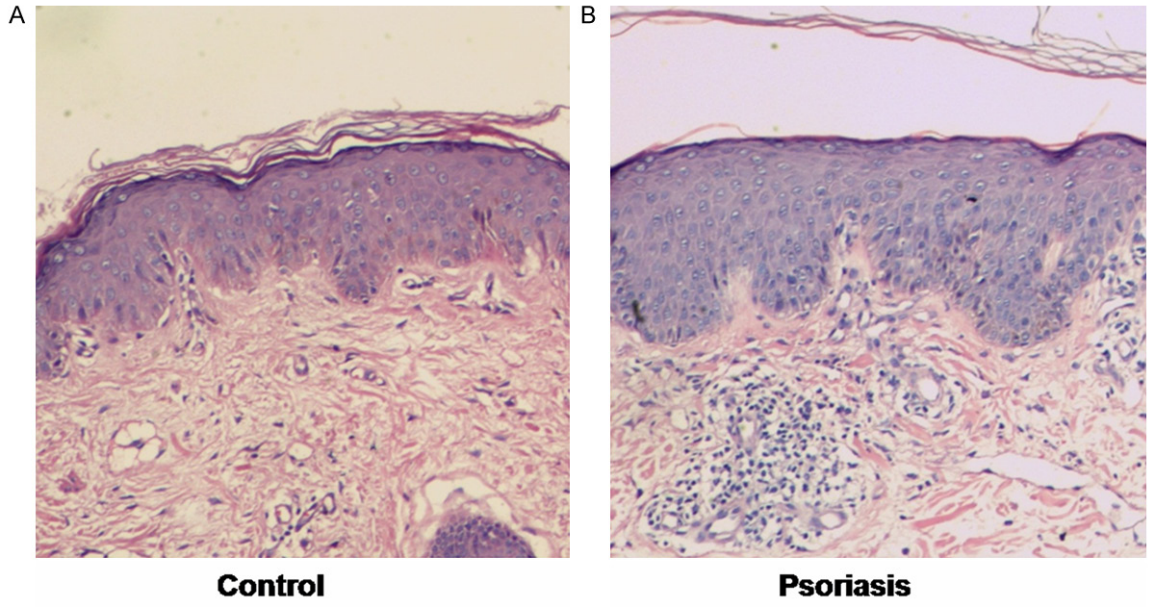

Figure 3. Infiltration of T lymphocytes to dermis of psoriasis patient. A. Normal skin tissue. B. Infiltration of T lymphocytes in skin tissues of patients with psoriasis. HE staining, Magnification, 100×.

an in healthy subjects (31.14±3.91 pg/mL, P=0.0051; **Figure 2A**). There was a positive correlation between PASI scores and CGRP levels (Spearman's correlation coefficient =0.352, P=0.0266; **Figure 2B**).

Effects of CGRP on T lymphocyte adhesion and migration

Excessive T lymphocyte infiltration is a key contributor to psoriatic lesions. As shown in **Figure 3**, the amount of T lymphocytes in psoriasis vulgaris skin is higher than in normal skin. The infiltration of T lymphocytes is involved in cell migration and adhesion to derma. We have observed the effects of CGRP on $CD3^+$ T lymphocyte adhesion and migration. $CD3^+$ T lymphocytes were identified by flow cytometry. CGRP at different concentrations promotes the adhesion and migration ability of T lymphocytes, which are inhibited by the presentation of specific CGRP receptor antagonist CGRP (8-37) (P=0.0085) (**Figure 4A** and **4B**).

To specify the cause of CGRP promotion of the adhesion and migration of T lymphocytes, we

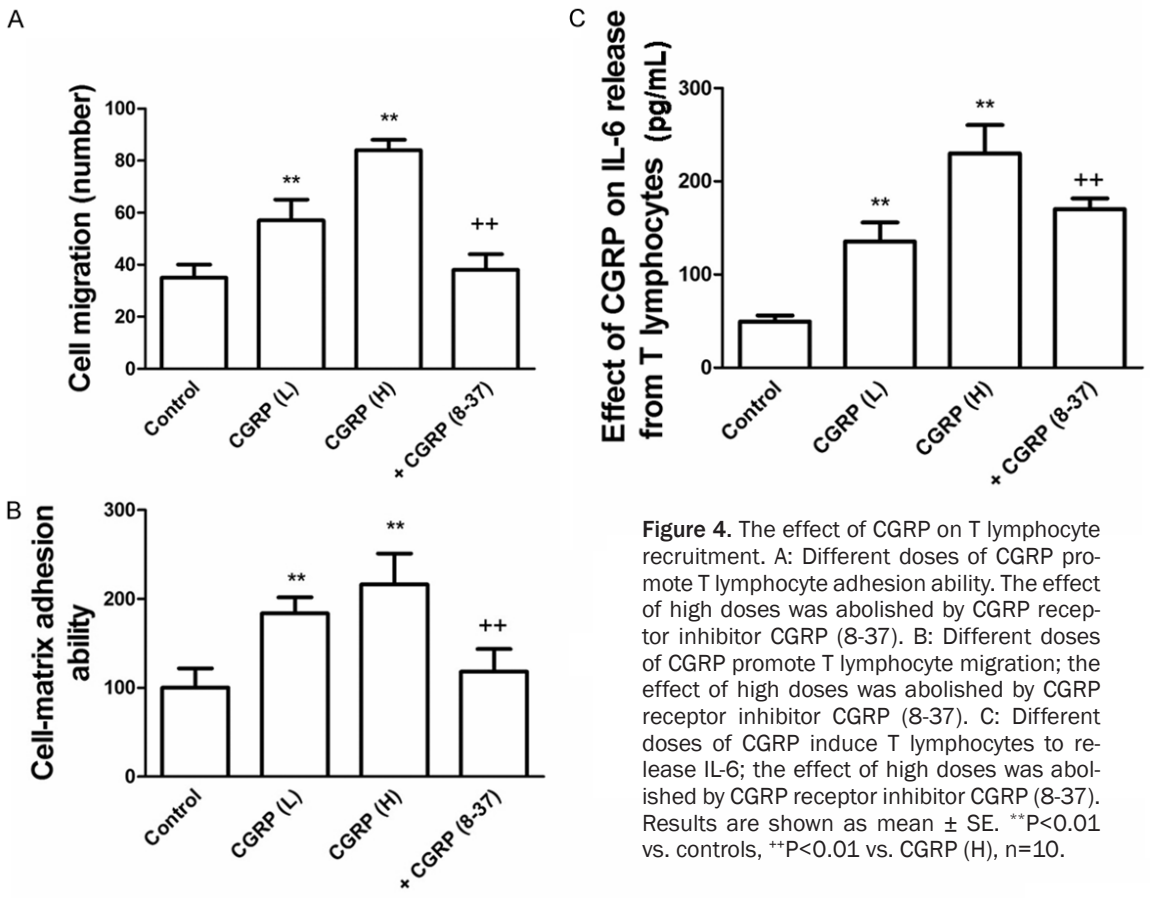

Figure 4. The effect of CGRP on T lymphocyte recruitment. A: Different doses of CGRP promote T lymphocyte adhesion ability. The effect of high doses was abolished by CGRP receptor inhibitor CGRP (8-37). B: Different doses of CGRP promote T lymphocyte migration; the effect of high doses was abolished by CGRP receptor inhibitor CGRP (8-37). C: Different doses of CGRP induce T lymphocytes to release IL-6; the effect of high doses was abolished by CGRP receptor inhibitor CGRP (8-37). Results are shown as mean ± SE. $^{**}P<0.01$ vs. controls, $^{++}P<0.01$ vs. CGRP (H), n=10.

observed IL-6 release from T lymphocytes treated with CGRP. CGRP significantly increased IL-6 release from T lymphocytes, which was abolished by CGRP (8-37) (P<0.0039) (**Figure 4C**).

Effects of acitretin on CGRP mRNA synthesis and release

The mRNA levels of both CGRP I and CGRP II extracted from T lymphocytes were increased in psoriasis vulgaris patients compared to healthy subjects. The synthesis of CGRP I and CGRP II was decreased in T lymphocytes from psoriasis patients treated with acitretin for 1 month (**Figure 5B** and **5C**), and the plasma CGRP level of patients with psoriasis vulgaris was correspondingly markedly decreased (P= 0.0058) (**Figure 5A**). To obtain direct evidence for an effect of acitretin on secretion from T lymphocytes, we observed T lymphocyte CGRP secretion from healthy subjects treated with different concentrations of acitretin. The results showed that acitretin may inhibit CGRP release from T lymphocytes in a dose-dependent manner (P=0.0071) (**Figure 5D**).

Discussion

CGRP, a 37-amino acid neuropeptide in the capsaicin-sensitive sensory nerves, is widely distributed in the central and peripheral nervous systems in humans [18]. It shows a variety of biological (or pathogenetic) effects on tissues, including tissues associated with the gastrointestinal, respiratory, endocrine, and peripheric nervous systems [19]. In the skin, the ability of CGRP to initiate cutaneous inflammation and induce keratinocyte proliferation is key to our interest in its putative role in psoriasis [3, 20].

CGRP has been found to be overexpressed in lesional psoriatic skin [21]. Despite many studies on the expression of this neuropeptide in psoriatic skin, little is known about the level of circulating CGRP in patients suffering from psoriasis. Our present study showed that the expression of CGRP in psoriasis patients (both

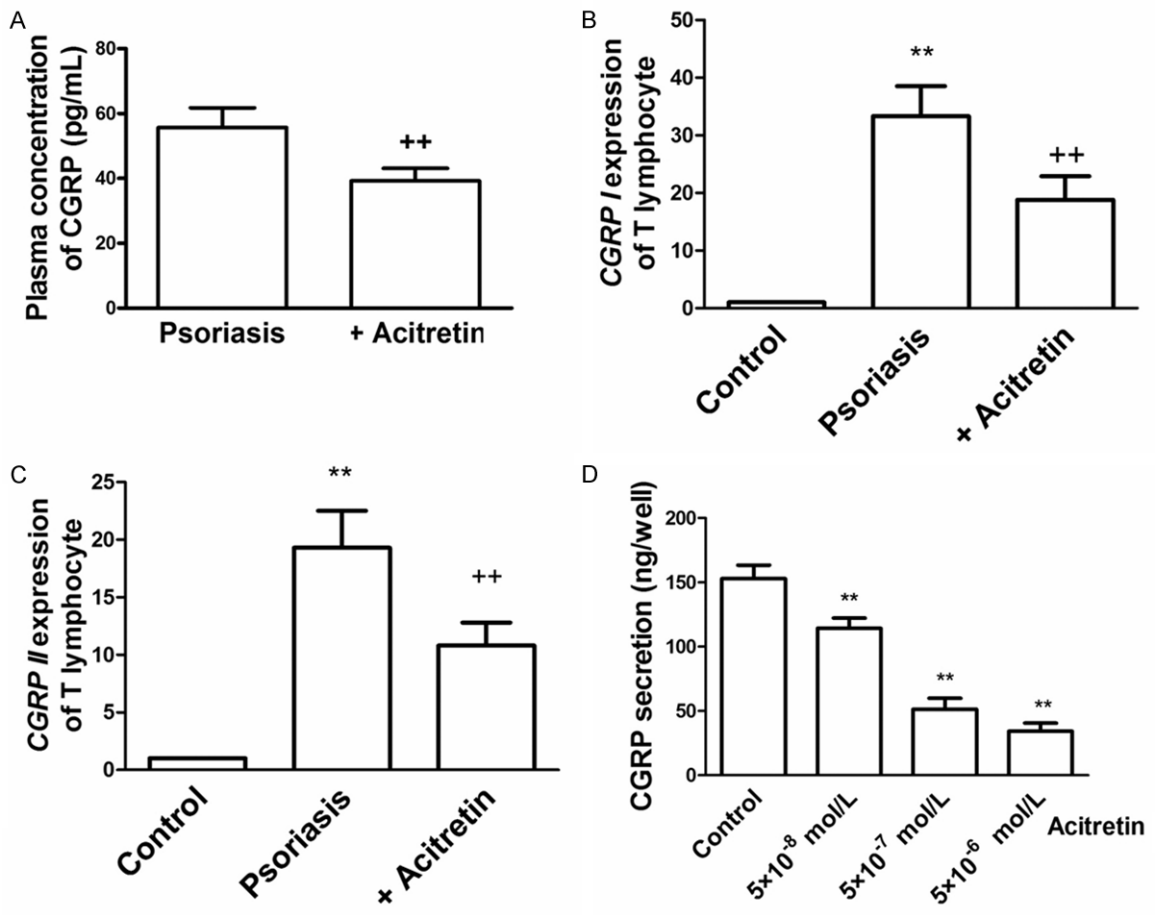

Figure 5. The effects of acitretin on CGRP synthesis and release in T lymphocytes. A: Change of serum CGRP levels of psoriasis vulgaris treated by acitretin. **P<0.01 vs. controls, n=12. B: Change of CGRP I mRNA level from T lymphocytes in psoriasis vulgaris treated by acitretin. **P<0.01 vs. controls, $^{++}$P<0.01 vs. psoriasis, n=12. C: Change of CGRP II level mRNA from T lymphocytes in psoriasis vulgaris treated by acitretin. Results are shown as mean ± SE. **P<0.01 vs. controls, $^{++}$P<0.01 vs. psoriasis, n=12. D: Change of T lymphocytes culture medium CGRP levels in the presence of acitretin **P<0.01 vs. controls, $^{++}$P<0.01 vs. psoriasis, n=14.

in skin and serum) is higher than its expression in the skin and serum of healthy individuals. Moreover, we found that levels of CGRP in patients with psoriasis were positively correlated with the results of PASI. These results indicate that the CGRP concentration in serum is likely to be a biomarker of psoriasis symptoms.

Psoriatic plaque is characterized by a marked infiltration of activated $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells infiltrate mainly the dermis, whereas $CD8^+$ T cells are present in the epidermis [22]. In the present study, $CD3^+$ T lymphocytes (both $CD4^+$ and $CD8^+$ T cells were included) of humans were accumulated to observe infiltration activation under different conditions. CGRP is able to activate many cells belonging to the immune system, including dendritic cells, lymphocytes, macrophages, and neutrophils [23, 24]. Our results showed that CGRP promotes T lymphocytes adhesion and migration *in vitro*, which may be inhibited by CGRP-receptor blocker CGRP (8-37). It was deduced that the infiltration ability of T lymphocytes is aggregated by circulating or located CGRP released from nearby cells.

It has been reported that neuropeptides also stimulate the synthesis and release of many proinflammatory cytokines from target cells such as lymphocytes, dendritic cells, and keratinocytes, inducing related tissue dysfunction [4, 25]. Our study showed that CGRP is able to promote the release of IL-6 from T lymphocytes; IL-6 is considered a proinflammatory cytokine that maintains the status of psoriasis. Our

results indicated that the effects of CGRP on the activity of T cells may be related to the promotion of secretion from cytokines.

CGRP may become an important therapy target in dermatological disease, because it exacerbates the inflammatory process [26]. For instance, treatment with capsaicin, a CGRP depleter, produces a significant therapeutic effect on psoriasis patients [27].

Acitretin is one of the most effective treatments for localized and pustular forms of psoriasis. The therapeutic mechanism of acitretin is not clear. Acitretin is known to reestablish a more normal pattern of cell growth and, to a lesser degree, to have some effect on immune cell processes [11]. It has been reported that 9-cis retinoic acid (an analogue of acitretin) decreases the release of CGRP in a dose-dependent manner in CA-77 C cells [12]. Similarly, retinoic acid (another analogue of acitretin) abolishes the CGRP autocrine system involved in the proliferation of F9 cells [13]. These results demonstrate that CGRP release may be reduced by acitretin or its analogue. Our results also indicate that CGRP mRNA levels from T cells and serum CGRP content were decreased in psoriasis patients continuously given acitretin for 1 month. It was also shown that acitretin may inhibit the secretion of endogenous CGRP from T lymphocytes. This evidence indicates that the treatment effect of acitretin is related to inhibition of CGRP secretion.

In summary, for the first time, we report that i) levels of CGRP in patients with psoriasis were correlated with their PASI score; ii) excessive infiltration of T lymphocytes in derma is related to high levels of circulating and/or skin CGRP; and iii) the therapeutic effects of acitretin are related to decreases in CGRP synthesis and release in psoriasis vulgaris. To obtain the precise cellular molecular mechanism involved in CGRP and acitretin treatment, more experiments are required.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiang Chen and Juan Su, Department of Dermatology, Xiangya Hospital, Central South University, Changsha, China. Tel: +86-731-84327128; Fax: +86-731-84327128; E-mail: chenxiangck@126.com (XC); 540020068@QQ.com (JS)

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