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Andrograpanin, a Compound Isolated From Anti-Inflammatory Traditional Chinese Medicine *Andrographis paniculata*, Enhances Chemokine SDF-1α-Induced Leukocytes Chemotaxis

Li-Li Ji,[1,2] Zhu Wang,[2] Fang Dong,[2] Wen-Bo Zhang,[2]* and Zheng-Tao Wang[1]**

[1]Department of Pharmacognosy, China Pharmaceutical University, 1 Shen Nong Road, Nanjing, People's Republic of China
[2]Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue, Yang Road, Shanghai, People's Republic of China

Abstract *Andrographis paniculata* is a traditional Chinese medicine (TCM) that has been effectively used for treatment of infection, inflammation, cold, fever, and diarrhea in China. However, mechanism of its therapeutic function is not well known. In the current study, we showed one of its components, andrograpanin, could enhance chemokine stromal cell-derived factor-1α (SDF-1α) induced chemotaxis in Jurkat and THP-1 cells. Further study demonstrated that this kind of effect was CXC chemokine receptor-4 (CXCR4) specific, since andrograpanin could not enhance other chemokines, such as RANTES, monocyte chemotactic protein-1 (MCP-1), etc. induced cell chemotaxis. Mechanisms of andrograpanin exerting its effect were not directly in the receptor and G protein coupling level because it had no effect on the binding of SDF-1 to CXCR4, SDF-1 induced G protein activation and adenyly cyclase inhibition. However, receptor internalization might be involved, since we found it significantly reduced SDF-1α-induced CXCR4 internalization. J. Cell. Biochem. 95: 970–978, 2005.

Key words: andrograpanin; chemokine; leukocyte; SDF-1α; CXCR4; chemotaxis; internalization

Chemokines, a family of structurally related low molecular mass cytokines, are crucial to the development of lymphoid tissue and the migration of leukocytes [Zlotnik et al., 1999]. Chemokines are distinguished based on the relative position of conserved residues into four subfamilies, designated as CC, CXC, C, and CX3C chemokines. Chemokines exert their biological effects by binding to chemokine receptors, which belong to the seven transmembrane G protein coupled receptor family [Horuk, 2001]. The migratory response of leukocytes to

Abbreviations used: RPMI 1640, Roswell Park Memorial Institute 1640; FBS, fetal bovine serum; PRC, People's Republic of China; SDF-1, stromal cell-derived factor; IL-8, interleukin-8; MIP-1, macrophage inflammatory protein-1; MCP-1, monocyte chemotactic protein-1; RANTES, regulated on activation normal T cell expressed; CXCR4, CXC chemokine receptor-4; andro, andrograpanin; and, andrographolide; cAMP, cyclic AMP; IBMX, 1-methyl-3-isobutylxanthine; PBLs, peripheral blood leukocytes; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; PTX, pertussis toxin; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; HIV, human immunodeficiency virus.

Li-Li Ji and Zhu Wang contributed equally to this work.

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*Correspondence to: Wen-Bo Zhang, PhD, Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai, 200031, PRC. E-mail: zhangwenbo@sibs.ac.cn

**Correspondence to: Zheng-Tao Wang, PhD, Department of Pharmacognosy, China Pharmaceutical University, 1 Shen Nong Road, Nanjing, 210038, PRC.
E-mail: herbs@mailbox.cpu.edu.cn

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chemokines forms the first line of host defense to the invaded microbial agents [Hedrick and Zlotnik, 1996; Luster, 2002]. Moreover, chemokines have also been shown to be involved in a number of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, dermatitis, asthma, organ transplant rejection, etc. [Horuk, 2001].

The CXC chemokine stromal cell-derived factor-1 (SDF-1), which exists in three splice variants, SDF-1α, SDF-1β, and SDF-1γ [Shirozu et al., 1995; Gleichmann et al., 2000], was originally isolated as a pre-B-cell stimulatory factor [Nagasawa et al., 1994]. SDF-1 selectively activates CXC chemokine receptor-4 (CXCR4), a chemokine receptor initially identified in leukocytes. SDF-1 and its cognate receptor CXCR4 have recently sparked substantial interests because of their roles in immune regulation, embryonic development, cancer metastasis, and human immunodeficiency virus (HIV) pathogenesis [Nagasawa et al., 1998; Tachibana et al., 1998; Zou et al., 1998; Stantchev and Broder, 2001; Libura et al., 2002]. SDF-1/CXCR4 signaling is critical for early human T-cell development and activation [Nanki and Lipsky, 2000; Hernandez-Lopez et al., 2002], B-cell lymphopoiesis [Ma et al., 1998], and brain inflammation [Odemis et al., 2002].

Andrographis paniculata of Acanthaceae Family is one of the Chinese herbs reputed to be effective in the treatment of infection, inflammation, cold, fever, and diarrhea in China [Chang and But, 1987]. However, mechanisms of its therapeutic functions are less known. Andrographolide, a major compound of *Andrographis paniculata*, has been reported to suppress the expression of inducible nitric oxide synthase [Chiou et al., 1998, 2000] and the production of neutrophil reactive oxygen species [Shen et al., 2000], which might be related with its anti-inflammatory effects. Neoandrographolide, another major compound of *Andrographis paniculata*, has been reported to reduce NO production, which plays an important role in inflammation [Batkhuu et al., 2002].

Andrograpanin is a minor compound of *Andrographis paniculata* and a hydrolysate from neoandrographolide in vivo and in vitro, the function of which has yet to be determined. In this study, we reported for the first time that andrograpanin selectively enhanced chemokine SDF-1α-induced leukocyte chemotaxis, which might contribute to the anti-infectious function of *Andrographis paniculata.*

MATERIALS AND METHODS

Chemicals and Reagents

Roswell Park Memorial Institute 1640 (RPMI1640) and fetal bovine serum (FBS) were purchased from GIBCO-BRL. SDF-1α, RANTES, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein-1 (MCP-1), interleukin-8 (IL-8), and monoclonal antibody agonist CXCR4 receptor (12G5) were all purchased from R&D. Andrograpanin, andropholide, and neoandrographolide were isolated from *Andrographis paniculata* in our lab in China Pharmaceutical University. [^{35}S] GTPγS (1250Ci/mmol) and [^{3}H] cyclic AMP (cAMP) were purchased from Amersham Pharmacia Biotech. FITC-conjugated affinity F(ab′)2 goat antimouse IgG was from Jackson lab. Transwell (5 μm) was from Corning Costar. All other reagents unless indicated were from Sigma Chemical Co. (St. Louis, MO).

Treatment Solutions

Andrographis paniculata Nees was collected in Linquan (Anhui Provine, China) in September 1998 and authenticated by Prof. Zhengtao Wang (China Pharmaceutical University, Nanjing, China). A voucher specimen was deposited in Herbarium of China Pharmaceutical University. The dried leaves (10 kg) was powdered and macerated in alcohol and percolated with eight volumes of alcohol. After removal of a half volume of the solvent under reduced pressure, the extract was decolored with activated charcoal and filtrated. The filtrate was concentrated to remove the alcohol and partitioned with ethyl acetate. The ethyl acetate soluble portion was recovered from solvent (450 g residue) and subjected to silica gel column and eluted with petroleum-acetone in gradient. The eluates were collected and monitored by thin layer chromatography, and similar fractions were combined. Andrograpanin was obtained from the petroleum eluates after recrystallization. Andrograpanin was structurally elucidated based on ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectral evidences. The structure was showed in Figure 1. The purity of the compounds was more than 98% as determined by high pressure liquid chromatography (HPLC) analysis.

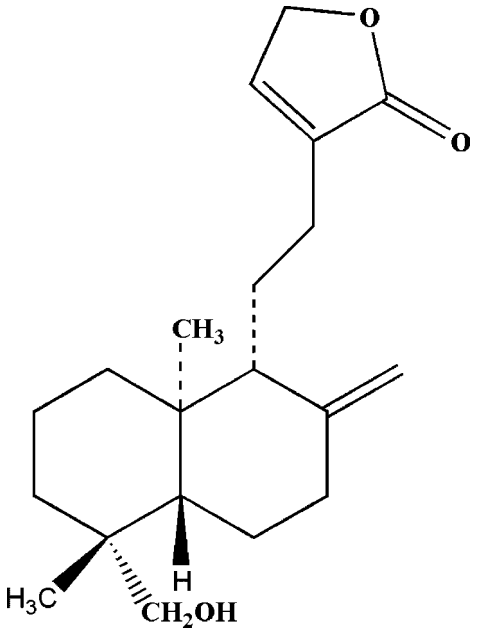

Fig. 1. The chemical structure of andrograpanin (andro.).

Cell Culture

Jurkat cell and THP-1 cell (American Type Culture Collection, ATCC) were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS (Gibco-BRL). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Transwell Chemotaxis

Cells in culture medium were centrifuged and then resuspended in RPMI 1640 containing 1 mg/ml BSA overnight in 5% CO_2 at 37°C. Cells (0.1 ml) at 6×10^6/ml pretreated with or without test reagents for 15 min at 37°C were added to the top chamber of a 24-well transwell (6.5-mm diameter, 5-μm pore size) and incubated for 2 h at 37°C in 5% CO_2. Cells passing through the membrane were counted by microscopy.

12G5 Binding Assay

Cells at the density of 2×10^6 cells/ml were pretreated with or without andrograpanin for 30min at 4°C, then 10 nM SDF-1α were added and incubated cell suspension for another 1 h at 4°C. After washed twice with cold phosphate-buffered saline (PBS), cells were incubated in the presence of CXCR4 antibody 12G5 for 1 h at 4°C without andrograpanin and SDF-1α. Then cells were washed twice with cold PBS and treated with FITC-conjugated affinity-purified goat anti-mouse IgG for 1 h at 4°C in dark. Cells were washed twice with cold PBS, resuspended in the PBS and analyzed by a FACSCalibur™ flow cytometer (Becton Dickinson) [Fenard et al., 2001].

[^{35}S] GTPγS Binding Assay

The assay was carried out as described [Zhang et al., 2003]. Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM EGTA at 4°C. After the lysate was centrifuged at 30,000*g* for 10 min, the membrane pellet was resuspended and aliquots containing 12 μg protein were pretreated with or without andrograpanin for 15 min, and then incubated at 30°C for 1 h in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 40 μM GDP, and 0.5 nM [^{35}S] GTPγS (1,200 Ci/mmol) in the presence or absence of the chemokines in a total volume of 100 μl. The reaction was terminated by adding cold PBS and filtering through GF/C filters. Radioactivity of each sample was measured in a liquid scintillation spectrophotometer. Data were means of duplicate samples. Basal binding was determined in the absence of agonist, and non-specific binding was obtained in the presence of 10 μM GTPγS. The percentage of stimulated [^{35}S] GTPγS binding was calculated as:

$$[\mathrm{CPM}_{\mathrm{sample}} - \mathrm{CPM}_{\mathrm{non-specific}}]/[\mathrm{CPM}_{\mathrm{basal}} - \mathrm{CPM}_{\mathrm{non-specific}}] \times 100.$$

cAMP Assay

Cells were pretreated with or without andrograpanin for 15 min, and then challenged with or without chemokines in the presence of 10 μM forskolin and 500 μM 1-methyl-3-isobutylxanthine (IBMX) at 37°C for indicated times. The reaction was terminated with 1 N perchloric acid and then neutralized with 2 M K_2CO_3. The cAMP level of each sample was determined using radioimmunoassay as described previously [Zhang et al., 2003]. The values were calculated as:

$$[\mathrm{cAMP}_{\mathrm{forskolin+agonist}} - \mathrm{cAMP}_{\mathrm{basal}}]/[\mathrm{cAMP}_{\mathrm{forskolin}} - \mathrm{cAMP}_{\mathrm{basal}}] \times 100$$

where $\mathrm{cAMP}_{\mathrm{forskolin+agonist}}$ is cAMP accumulation in the presence of forskolin and chemokine, and $\mathrm{cAMP}_{\mathrm{basal}}$ is cAMP accumulation in the absence of forskolin and chemokine, and $\mathrm{cAMP}_{\mathrm{forskolin}}$ is cAMP accumulation in the presence of forskolin alone.

SDF-1α Induced Internalization of CXCR4 Receptor

Cells at the density of 2×10^6 cells/ml were pretreated with or without andrograpanin for 30 min at 37°C, then 3 nM SDF-1α was added and incubated cell suspension for another 30 min at 37°C. At the end of incubation, cells were washed twice with cold PBS. Cells were then exposed to the CXCR4 antibody 12G5 for 1 h at 4°C in the absence of SDF-1α and andrograpanin. After washed twice with cold PBS, cells were treated with FITC-conjugated affinity-purified goat anti-mouse IgG for 1 h at 4°C in dark. Then cells were washed twice with cold PBS, resuspended in the PBS, and analyzed by a FACSCalibur™flow cytometer (Becton Dickinson).

Statistical Analysis

All values were expressed as means ± standard error of the mean (SEM). Student's *t*-test was used to determine the significance of differences in multiple comparisons. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Andrograpanin, but not Andrographolide and Neoandrographolide Enhanced SDF-1α-Induced Jurkat Cells Chemotaxis

Chemotaxis is the prototypic function of chemokines, and always serves as a biologically relevant functional in vitro assay for chemokine receptor activation [Campbell et al., 1996]. Jurkat cells, a human T-leukemia cell line,

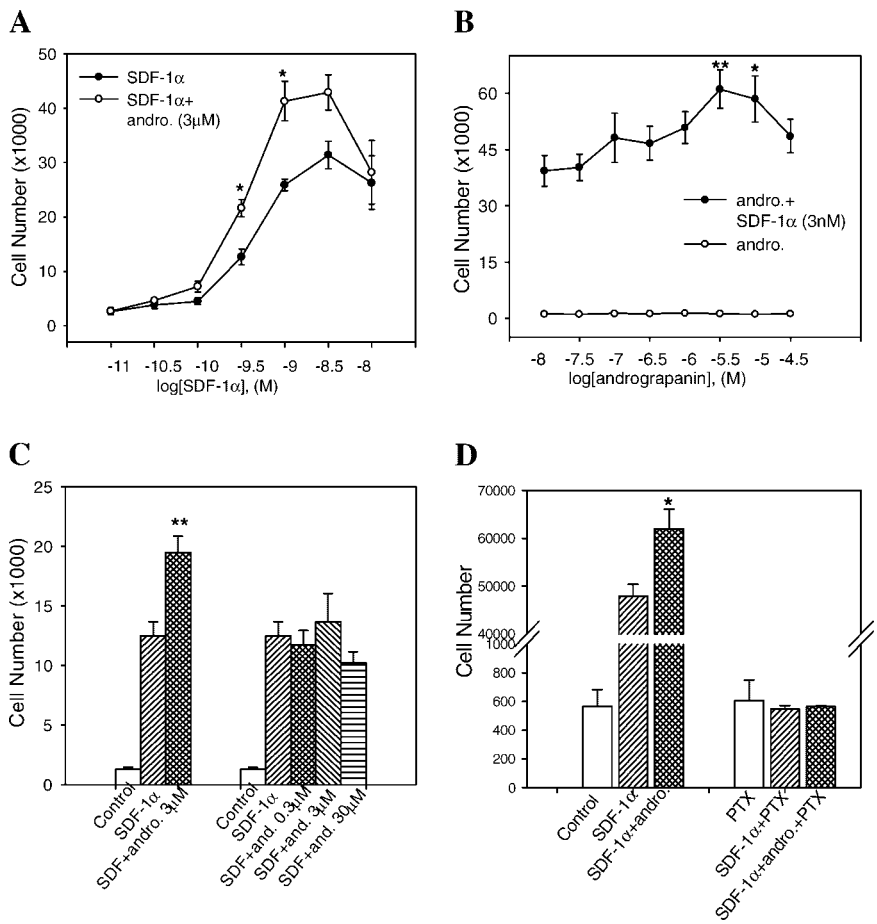

Fig. 2. Enhancement of SDF-1α-induced chemotaxis by andrograpanin (andro.) in Jurkat cells. **A**: Cells were pretreated with or without 3μM andrograpanin for 15 min at 37°C and incubated with SDF-1α at various concentrations at 37°C for 2 h. **B**: Cells were pretreated with andrograpanin at various concentrations for 15 min at 37°C and incubated in the presence or absence of 3nM SDF-1α at 37°C for 2 h. **C**: Cells were pretreated with andrograpanin (andro.) or andrographolide (and.) for 15 min at 37°C and incubated with 3 nM SDF-1α at 37°C for 2 h. **D**: Cells were pretreated with or without andrograpanin (andro.) for 15 min at 37°C, and incubated with or without 150 ng/ml PTX for 2 h at 37°C, and then incubated in the presence or absence of 3 nM SDF-1α at 37°C for 2 h. The migrated cells were collected and counted as described above. $^*P < 0.05$, $^{**}P < 0.01$ compared with absence of andrograpanin. Data were mean ± SE of three independent experiments performed in duplicate.

which express abundant CXCR4 receptor (data not shown), could chemotaxis upon exposure to SDF-1α. As shown in Figure 2A, a classic bell-shape chemotactic response was observed when Jurkat cells were exposed to increasing concentrations of SDF-1α. Interestingly, pretreatment of Jurkat cells with 3 μM andrograpanin significantly enhanced their chemotaxis induced by SDF-1α (Fig. 2A). The effect of andrograpanin was dependent on SDF-1α because it alone did not elicit any significant Jurkat cells chemotaxis. Andrograpanin enhanced SDF-1α-induced chemotaxis with an optimal concentration at about 3 μM, while the effect of higher dose of andrograpanin was weak, which is similar to trichosanthin (TCS) enhanced chemokine-induced cell chemotaxis [Zhao et al., 1999] (Fig. 2B). We also tested the cytotoxicity of andrograpanin on Jurkat cells, and we did not observed significant toxic effect of andrograpanin on cell (data not shown).

Since it has been reported that two components of *Andrographis paniculata*, andrographolide and neoandrographolide, had significant anti-inflammatory function [Chiou et al., 1998, 2000; Shen et al., 2000], we also tested their effects. However, neither andrographolid (Fig. 2C) nor neoandrographolide (data not shown) had significant effect on SDF-1α-induced Jurkat cells chemotaxis.

Chemokine receptor CXCR4 is predominantly coupled by pertussis toxin (PTX) sensitive Gi/o protein, so we observed the effect of PTX, which can block Gα protein activation [Bokoch et al., 1982], on Jurkat cells chemotaxis. As shown in Figure 2D, PTX blocked SDF-1α and SDF-1α/andrograpanin induced Jurkat cells chemotaxis including andrograpanin enhanced SDF-1α-induced cells chemotaxis.

Andrograpanin Enhanced SDF-1α-Induced Cell Chemotaxis but Had No Effect on Other Chemokines-Induced Chemotaxis

So far, 18 chemokine receptors and more than 50 chemokines have been reported [Chen et al., 2004]. To test the specificity of andrograpanin, its effect was tested by using of several representative chemokine such as CC chemokines RANTES, MIP-1α, MCP-1, and CXC chemokine IL-8 in two other leukocytes, PBLs and THP-1. Andrograpanin significantly enhanced THP-1 cells (Fig. 3) and PBLs (data not shown) chemotaxis induced by SDF-1α but had no effect on chemotaxis induced by other chemokines. Our results demonstrate the effect of andrograpanin is CXCR4 specific.

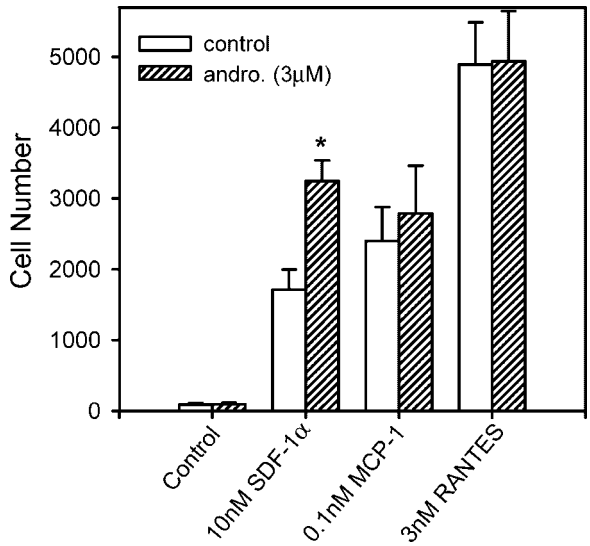

Fig. 3. The enhancement effects of andrograpanin on chemokine-induced chemotaxis in THP-1 cells. THP-1 cells were pretreated with 3 μM andrograpanin for 15 min at 37°C, and then incubated with SDF-1α, RANTES, MCP-1 at 37°C for 2 h. The migrated cells were collected and counted as described above. $*P < 0.05$ compared with absence of andrograpanin. Data were mean ± SE of three independent experiments performed in duplicate.

Andrograpanin Did Not Affect SDF-1 Binding to CXCR4 and CXCR4 Mediated G Protein Activation

Our previous study demonstrated that activation of chemokine receptors could induce Gi/o proteins activation and subsequently inhibition of adenyly cyclase. Trichosanthin (TCS), an active protein component isolated from a traditional Chinese medicinal herb *Trichosanthes kirilowii*, has been shown to augment chemokine-stimulated activation of chemokine receptors CCR5 and CXCR4 through its interaction with chemokine receptors [Zhao et al., 1999]. However, in the currently study, we did not find andrograpanin affect the binding of SDF-1α with CXCR4 because the reduced binding of 12G5, a CXCR4 receptor conformation-dependent monoclonal antibody, to CXCR4 by SDF-1α did not change in the presence of andrograpanin (Fig. 4A). In addition, andrograpanin did not enhance 10 nM SDF-1α-stimulated G protein activation (Fig. 4B) in GTPγS binding assay. We also did not observe the significant effect of andrograpanin on SDF-1α-induced adenylyl cyclase inhibition in cAMP

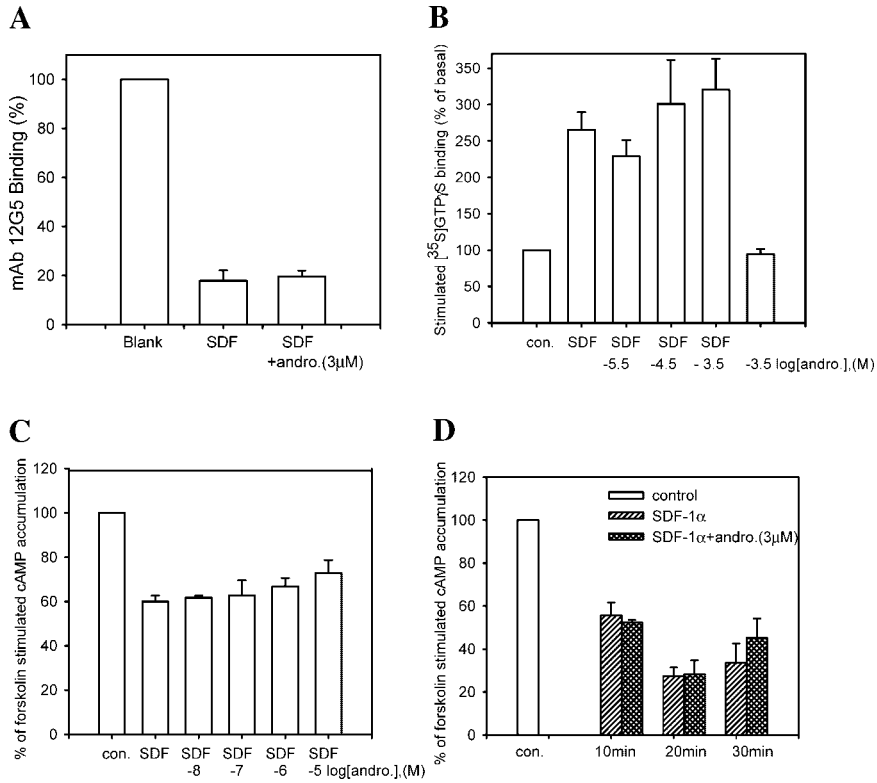

Fig. 4. The effects of andrograpanin on SDF-1α binding to CXCR4 receptor and G protein activation in Jurkat cells. **A**: Cells were pretreated with or without 3 μM andrograpanin for 30 min at 4°C and incubated in the presence or absence of 10 nM SDF-1α and 12G5 antibody for 60 min at 4°C. After several washes, the binding of 12G5 antibody was detected using FITC-conjugated secondary antibody. The percentage of positive cells was determined using a FACS scan analyzer. **B**: The cell membranes were pretreated with andrograpanin for 15 min and stimulated by 10 nM SDF-1α at 30°C for 60min. The [^{35}S] GTPγS binding of each sample was measured as described in Materials and Methods. **C**: Jurkat cells were pretreated with andrograpanin for 15 min and stimulated by 10 nM SDF-1α at 37°C for 10 min. **D**: Jurkat cells were pretreated with andrograpanin for 15 min and stimulated by 10 nM SDF-1α at 37°C for indicated times. The cellular cAMP level was measured as described in Materials and Methods. Data were mean ± SE of three independent experiments performed in duplicate.

formation assay (Fig. 4C,D). Our results clearly showed that the effect of andrograpanin was independent of SDF-1 bind to CXCR4 and G protein activation.

Andrograpanin Reduced SDF-1α-Induced CXCR4 Receptor Internalization

It has been shown that receptor internalization is involved in chemotaxis [Guinamard et al., 1999; Yang et al., 1999; Kraft et al., 2001; Ding et al., 2003]. We tested if andrograpanin could affect SDF-1α-induced receptor internalization. As shown in Figure 5A, 3 nM SDF-1α induced significant internalization of CXCR4 receptor in Jurkat cells thus may contribute to the stop of CXCR4 receptor mediated signal. Although andrograpanin itself had no effect on surface CXCR4 expression, pretreatment of Jurkat cells with andrograpanin for 30 min significantly reduced SDF-1α induced internalization of CXCR4 receptor. The effect of andrograpanin was further confirmed in the THP-1 cells, as shown in Figure 5B, 3 μM andrograpanin significantly reduced SDF-1α-induced CXCR4 receptor internalization in THP-1 cells.

DISCUSSION

Traditional Chinese medicine (TCM) represents one of the oldest medical approaches in the world and has been used for centuries in China [Ergil, 1996]. As complementary and alternative medicine (CAM) has been becoming more and more popular and attractive in Western society, there are increasing interest arisen on elucidating the efficacy, safety, and functional mechanism of TCM. However, so far, there are few herbal medicines whose functional mechanisms are well known. In

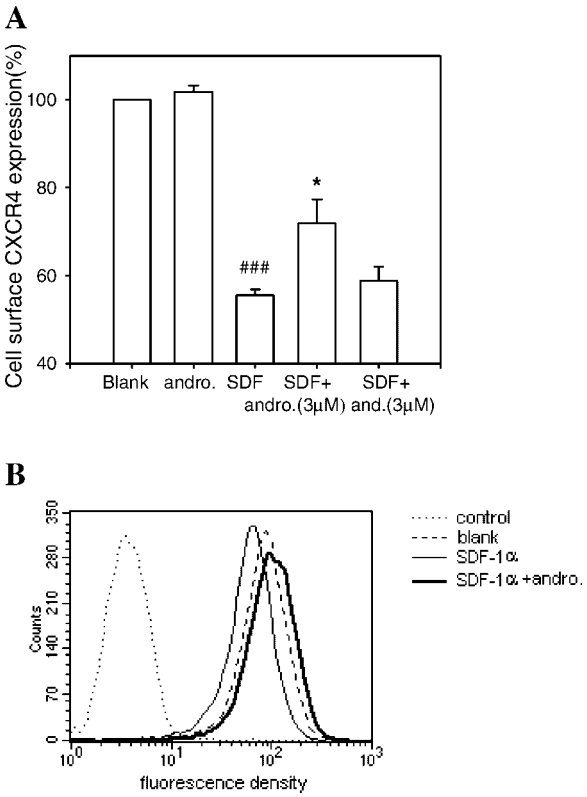

Fig. 5. Andrograpanin reduced SDF-1α-induced CXCR4 receptor internalization. **A**: Jurkat cells were pretreated with or without 3 μM andrograpanin for 30 min at 37°C and incubated in the presence or absence of 3 nM SDF-1α for 30 min at 37°C. Cells were then stained with 12G5 antibody at 4°C and detected with FITC-conjugated secondary antibody using a FACS scan analyzer. The percentage of CXCR4 surface expression was calculated from the relative fluorescence intensity. $^{###}P < 0.001$ compared with absence of SDF-1α. $^{*}P < 0.05$ compared with absence of andrograpanin. Data were mean ± SE of three independent experiments. **B**: THP-1 cells were pretreated with or without 3 μM andrograpanin for 30 min at 37°C and incubated in the presence or absence of 3 nM SDF-1α for 30 min at 37°C, cells were then stained with 12G5 antibody at 4°C and detected with FITC-conjugated secondary antibody. FITC-labeled fluorescence signals on cell surface were analyzed by flow cytometry.

the current study, we found andrograpanin, one of components of *Andrographis paniculata*, could enhance SDF-1α-induced leukocytes chemotaxis. Although *Andrographis paniculata* has been reported for treating cancer in Asia [Matsuda et al., 1994], our finding also reminds us that it should be carefully applied to be used to treat cancer patients because it might induce tumor cell metastasis.

Chemokines play important roles in immune response by their abilities to induce directional migration and activation of leukocytes. It has been found that many anti-inflammatory TCMs functions are related with chemokine pathway. Baicalin, a compound isolated from medicinal plant *Scutellaria baicalensis* Georgi, exhibits anti-inflammatory activity by binding to chemokines [Li et al., 2000]. Shikonin, a compound from anti-inflammatory medicinal plant *Lithospermum erythrorhizon*, has been reported to be a CCR1 receptor antagonist [Chen et al., 2001]. The anti-inflammatory effect of Shuanghuanglian and Qingkailing, two multi-components of traditional Chinese medicinal peparations, exert their effects through suppressing the production of cytokines and chemokines by inhibition of NF-κB-regulated genes transcription [Chen et al., 2002]. In our study, we found andrograpanin, isolated from medicinal plant *Andrographis paniculata*, could modulate the chemokine pathway. However, in contrast to those inhibitory effects on chemokine pathway, our results showed that andrograpanin significantly enhanced chemokine SDF-1α-induced Jurkat, THP-1 and PBL cells chemotaxis, which indicated that the effect of andrograpanin might contribute to the anti-infectious function of *Andrographis paniculata*. As other two compounds isolated from *Andrographis paniculata*, andrographolide and neoandrographolide, have anti-inflammatory function through NO signal pathway, maybe andrograpanin and these two compounds cooperate with each other, which play important functions in theraputic effects of *Andrographis paniculata* in diseases of infection combined with inflammation. Moreover, our results also showed a specificity of andrograpanin in chemokine pathway since it had no effect on other chemokines RANTES, MCP-1, IL-8, and MIP-1α-induced chemotaxis.

The mechanisms that andrograpanin augmented SDF-1-induced chemotaxis are not clear. Molecules, which interact with CXCR4 receptor, SDF-1α, G proteins, or downstream signals, are all involved in regulating SDF-1α and CXCR4-mediated chemotaxis. However, in our study, andrograpanin did not affect SDF-1 binding to CXCR4, CXCR4-mediated G protein activation, and subsequent inhibition of cAMP formation. Receptor internalization has been shown to be involved in CXCR4-mediated cell chemotaxis. It has been reported that B-cell antigen receptor stimulation results in the inhibition of SDF-1α-induced migration through promoting PKC-dependent internalization of CXCR4 receptor [Guinamard et al., 1999]. L-selectin inhibits SDF-1-induced CXCR4 internalization and increases SDF-1-induced

lymphocyte adhesion as well as transendothelial migration [Ding et al., 2003]. Furthermore, upregulation of CXCR4 cell surface expression is required for the induction of SDF-1-dependent chemotaxis in several other cell types such as endothelial and dendritic cells [Gupta et al., 1998; Lin et al., 1998]. In our study, we found andrograpanin could reduce SDF-1α-induced CXCR4 receptor internalization. Our results suggest the enhanced chemotaxis of andrograpanin may be partly due to its inhibition of SDF-1α-induced CXCR4 receptor internalization. Many cellular signal molecules have been reported to be involved in SDF-1α/CXCR4 signal pathway, such as p38 MAPK [Misse et al., 2001], RasGAP-associated docking protein p62Dok-1 [Okabe et al., 2004], LIM kinase 1 [Nishita et al., 2002], ZAP-70 protein [Ticchioni et al., 2002], etc. Therefore, further investigation is needed to fully elucidate the precise mechanism of andrograpanin's effect on SDF-1α-induced chemotaxis.

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