Effects of Shikonin on the Proliferation and Activation of T Lymphocytes

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Abstract

Objective: To investigate the effects of Chinese herbal monomer shikonin which has the function of cooling blood and detoxification on abnormally activated T lymphocyte and its related signal pathways and to elucidate the role of shikonin in the pathological state of psoriasis and its mechanism of treating psoriasis. Materials and Methods: Jurkat E6-1 T lymphocytes were activated with phorbol ester and ionomycin. The shikonin concentration of 0.5-2 μg/mL was applied to the cells, and the proliferation of T lymphocytes was detected by Cell Counting Kit-8 assay. Flow cytometry was used to measure CD69 expression on the cell membrane and intracellular free calcium ion concentration ([Ca²⁺]i); enzyme-linked immunosorbent assay was used to detect the levels of interleukin (IL)-2, interferon-γ (IFN-γ), and (TNF-α) released by activated T lymphocytes; quantitative polymerase chain reaction and Western blot were used to observe the expression of nuclear transcription factor mRNA and protein, respectively. Results: Different concentrations of shikonin could significantly inhibit cell proliferation, CD69 expression, and secretion of Th1 cytokines. In addition, shikonin could effectively reduce the [Ca²⁺]i and protein kinase C phosphorylation proteins. Besides that, shikonin could significantly reduce the nuclear transcription factor nuclear factor of activated T lymphocytes mRNA expression, downregulate e-Jun mRNA and protein expression, and inhibit NF-κB protein expression. All the above indicators show a certain dose–effect relationship. Conclusions: Shikonin can exert immune regulation by inhibiting the function of overactivated T lymphocytes. Hence, this study provides experimental basis for the mechanism and application prospect of shikonin in the treatment of psoriasis.

Keywords: psoriasis, shikonin, signal transduction pathway, T lymphocyte activation, treatment principle of cooling blood and detoxification

Introduction

Psoriasis, called Baibi in traditional Chinese medicine (TCM), is a common clinical relapsing disease. It belongs to an autoimmune disorder disease under a certain genetic background. Abnormally activated T lymphocytes and the cytokines they release are the key sections for the occurrence of psoriasis. What is believed in TCM that the main pathogenesis of psoriasis is toxins accumulating in blood and heat toxins entering blood and damaging the collaterals, so the treatment principles mostly focus on cooling blood and resolving toxins. Under the guidance of this thought, Liangxue Huoxue Capsule as a hospital preparation for the treatment of psoriasis in blood-heat syndrome is created by Beijing Hospital of Traditional Chinese Medicine (Beijing, China). Our team conducted long-term and in-depth verification and research on the clinical efficacy of Liangxue Huoxue Capsule and proved its safety and effectiveness in the treatment of psoriasis in blood-heat syndrome at advantages of not causing stasis using herbs with functions of cooling and invigorating the blood, but not damaging the healthy qi using the herbs with functions of resolving toxins and enriching yin. Clinical studies have confirmed that Liangxue Jiedu Decoction can significantly improve the symptoms of erythema, infiltrative...
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Materials and Methods

Drug preparation
Shikonin (China Pharmaceutical and Biological Products Institute, Beijing, China) is dissolved in dimethyl sulfoxide (DMSO) and then diluted with ethanol. Before using, the DMSO concentration of solution is diluted to 1% or less and the ethanol concentration is diluted to <10% by phosphate-buffered solution. If shikonin is precipitated during dilution, ultrasound is used for its complete dissolution.

Shikonin concentration screening
Jurkat E6-1 T lymphocytes (Cell Resource Center, Peking Union Medical College Hospital, Beijing, China) were seeded in 96-well plate at 1 × 10^4 cells/well. 2 × 10^-7 mol/L phorbol 12,13-dibutyrate (PDB) (P8139, Sigma-Aldrich, Inc., St. Louis, MO, USA) and 5 × 10^-7 mol/L ionomycin (IM) (I9657, Sigma-Aldrich, Inc., St. Louis, MO, USA) were used to stimulate and activate cells. Meanwhile, shikonin at final concentrations of 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL, 2 μg/mL, and 4 μg/mL were treated to cells at 37°C, 5% CO₂ incubator (MYO-20AIC, SANYO, Osaka, Japan) for 48 h. Four hours before detection, each well was added with 10 μL Cell Counting Kit-8 (CCK-8) (JG659, Dojin Chemical Institute, Japan), and absorbance was measured at a wavelength of 450 nm by a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA, USA). A concentration range that is nontoxic to normal cells and has a certain pharmacodynamic effect is selected as the application dose for subsequent experiments.

Cell Counting Kit-8 assay
T cells were seeded in a 96-well plate at 1 × 10^4 cells/well. 2 × 10^-7 mol/L PDB (final concentration) and 5 × 10^-7 mol/L IM (final concentration) were used to stimulate and activate cells. Meanwhile, the cells were treated with a determined dose of shikonin above at 37°C and 5% CO₂ for 48 h. Four hours before the assay, 10 μL CCK-8 was added to each well, and absorbance was measured at a wavelength of 450 nm using a microplate reader. Each group had 6 wells and the experiment was repeated three times.

Flow cytometry detection

Flow cytometry detection of CD69 expression
Jurkat E6-1 T lymphocytes were seeded in a 12-well plate at 3 × 10^4 cells/well and treated with PDB, IM, and shikonin for 24 h in the same way as above. Cells were harvested and incubated with 10 μL anti-CD69-PE (341652, BD Bioscience, New Jersey, USA) for each sample at 4°C for 30–40 min in the dark. The expression rate of CD69-positive cells was detected via flow cytometry (FACSCalibur, BD Bioscience, New Jersey, USA). Each group had two wells and the experiment was repeated three times.

Concentration of free Ca²⁺
Cells were seeded in a 6-well plate at 7.5 × 10^4 cells per well and treated with PDB, IM, and shikonin for 16 h in the same way as above. And then, the cells were collected and 100 μL Fluo-3AM (5 μmol/L) was added and incubated at 37°C for 30 min in the dark. The unbound dye was washed away and the fluorescence intensity of intracellular free Ca²⁺ was measured by flow cytometry. Each group had duplication and the experiment was repeated three times.

Enzyme-linked immunosorbent assay
Cells were treated with PDB, IM, and shikonin in the same way as above. The cell supernatants which were collected after centrifugation were added to enzyme-linked immunosorbent assay kit (Bender, IFN-γ: BMS228HS, IL-2: BMS221HS, TNF-α: BMS223HS) to measure the levels of cytokines strictly in accordance with the instructions. The cell lysates were harvested to detect protein kinase C (PKC) phosphorylated protein content.

Real-time quantitative polymerase chain reaction
Cells were treated with PDB, IM, and shikonin in the same way as above. After cell collection, the total cellular mRNA was extracted and reverse transcribed, and the experimental procedures were performed following product instructions. Using SYBR Green (Qiagen, Duesseldorf, Germany) labeling, ABI 7500 (ABI7500, Thermo Fisher Scientific, Waltham, MA, USA) fluorescence quantitative polymerase chain reaction instrument was used to detect 40 cycles, and 2^-ΔΔCT was used for relative quantitative analysis of the target genes. Each group had two wells and the experiment was repeated three times.

All primers were synthesized by Sangon Biotech (Shanghai, China) [Table 1].

Western blot analysis
Cells were treated with PDB, IM, and shikonin in the same way as above. The cells were collected by centrifugation, lysed, and extracted for the total proteins. The protein quantification was determined by Bicinchoninic acid (BCA). The electrophoresis of the protein in the polyacrylamide gel was electrophoretically transferred to the polyvinylidene

area, and pruritus of the affected skin lesions in patients with a total effectiveness rate of 69.23%[3] and can markedly reduce the levels of IL-17, IL-1β, IFN-γ, IL-6, TNF-α, and vascular endothelial growth factor (VEGF) in peripheral blood of psoriatic patients in blood-heat syndrome,[4,6] reflecting some immunosuppression regulation. Preliminary basic research shows that high, moderate, and low doses of Liangxue Huoxue Capsules and its component herbs can obviously inhibit the abnormal activation of T lymphocytes, thereby regulating the progress of inflammatory reactions, and exert a certain immunosuppressive effect.[7,8] Arnebia root (Radix Arnebiae) is a chief herb in Liangxue Huoxue Capsule and has multiple functions of cooling blood, invigorating blood, and resolving toxins. In order to further study the potential mechanism of herbs with functions of cooling blood and resolving toxins on psoriasis, in this study, we selected shikonin, the main active ingredient of arnebia root as the research object, and endeavored to observe its effect on proliferation, activation, cytokine release, and relevant signal transduction pathways of activated T lymphocytes.
Effects of shikonin on the proliferation and activation of T lymphocytes

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Effect of shikonin on activated T lymphocyte activity

The experimental results showed that 4 μg/mL shikonin reduced the activity of T lymphocytes below the normal level, indicating its cytotoxicity to the cells and displayed a significant difference compared with the control group (P < 0.05). The shikonin at concentrations of 0.5–2 μg/mL all inhibited the activity of T lymphocytes stimulated by PDB + IM and reduced cell proliferation rates with inhibition rates of 26.45%, 26.45%, and 31.51%, respectively (all P < 0.01 vs. the model group). Whereas, 0.25 μg/mL shikonin did not show an inhibitory effect on activated T lymphocytes (vs. the model group, P > 0.05) [Table 2]. 0.5–2 μg/mL shikonin could be selected in the subsequent experiments.

Statistical analysis

Data are presented as mean ± standard deviation using SPSS 22.0 for statistical analysis. One-way analysis of variance was used for comparison between groups. P < 0.05 was considered statistically significant.

RESULTS

Effect of shikonin on activated T lymphocyte activity

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Effect of shikonin on expression of T cell activation marker CD69

In this experiment, 0.5–2 μg/mL shikonin was applied to T lymphocytes activated by PDB and IM, which showed that each concentration of shikonin could significantly reduce the expression of CD69 on the surface of T lymphocytes (P < 0.01 vs. model group) with the inhibition rates of 38.44%, 61.18%, and 65.7%, respectively, at an average reduction of 55%. Among them, the most significant inhibition was 2 μg/mL, followed by 1 μg/mL and 0.5 μg/mL shikonin in certain dose–effect manners [Figure 1 and Table 3].

Effect of shikonin on secretion levels of IFN-γ, interleukin-2, and tumor necrosis factor-α of activated T cells

During experiments, 0.5–2 μg/mL shikonin which were treated to activated T lymphocytes significantly reduced IFN-γ secretion, with a decrease of 41.88%, 61.51%, and 68.63% (all P < 0.01 vs. model group), respectively, in certain dose–effect manners. Among them, the downregulation was the most significant in 2 μg/mL shikonin, but its content was still higher than the inactivated cells (P < 0.05 vs. control group) [Table 4]. In addition, shikonin significantly decreased the ability of activated T lymphocytes to secrete IL-2 (P < 0.05, 0.01, and 0.01 vs. model group, respectively). The downregulation of 2 μg/mL shikonin was the most obvious, with the inhibition rate being up to 74.74%, which effectively inhibited the overrelease of cytokines. The inhibition rates of IL-2 by shikonin at concentrations of 0.5 μg/mL and 1 μg/mL were 40.82% and 50.83%, respectively, inferior to that of 2 μg/mL. The higher the drug concentration is, the stronger the inhibitory effect exerts [Table 4]. Moreover, different concentrations of shikonin significantly inhibited the TNF-α secretion of activated T lymphocytes, and the inhibition rates were 19.63%, 23.55%, and 49.34%, respectively (P < 0.05, 0.05, and 0.01 vs. model group). The higher the drug concentration is, the higher the inhibition rate becomes. The inhibitory effect was the most obvious at 2 μg/mL shikonin. The level of TNF-α in this group was comparable to that in the control group (P > 0.05 vs. control group), indicating that the concentration of the drug had a good inhibition of TNF-α secretion and regulated the cells to the preactivation level. Shikonin at concentrations of 0.5 μg/mL and 1 μg/mL could effectively reduce the level of TNF-α secreted by T lymphocytes, but the content was still high compared with the control group (P < 0.05) [Table 4].

Effect of shikonin on changes of free [Ca2+]i in activated T lymphocytes

The results showed that the fluorescence intensity of Ca2+ in activated T lymphocytes was significantly increased and the Ca2+ peak was shifted to the right. The concentration of intracellular free Ca2+ increased by nearly 300% compared with the resting state after activation (P < 0.01 vs. control group). The Ca2+ concentrations of cells in 0.5 μg/mL, 1 μg/mL, and 2 μg/mL shikonin groups were lower than that of those in the model group, with the inhibition rates of 18.18%, 31.11%,

Table 1: Primer sequences and product sizes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
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<tr>
<td>NF-ATc1</td>
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<td>5’-CAG GAT TCC GGC ACA GTC AT</td>
</tr>
<tr>
<td>c-Jun</td>
<td>5’-CTC AGA CAG TGC CCG AGA TG</td>
<td>5’-GCC GCG TTA GCA TGA GTT GG</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG</td>
<td>5’-TGA CGG GTG TAC CCC CAC GCC CAT CTA</td>
</tr>
</tbody>
</table>

Table 2: Effect of shikonin on activated T lymphocytes activities (n=6, x±s)

<table>
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<tr>
<th>Group</th>
<th>Dose</th>
<th>OD</th>
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<tr>
<td>Control</td>
<td></td>
<td>1.454±0.172**</td>
</tr>
<tr>
<td>Model</td>
<td></td>
<td>2.136±0.137***</td>
</tr>
<tr>
<td>Shikonin</td>
<td>0.25 μg/mL</td>
<td>2.051±0.495***</td>
</tr>
<tr>
<td></td>
<td>0.5 μg/mL</td>
<td>1.571±0.370***</td>
</tr>
<tr>
<td></td>
<td>1 μg/mL</td>
<td>1.517±0.411***</td>
</tr>
<tr>
<td></td>
<td>2 μg/mL</td>
<td>1.463±0.454***</td>
</tr>
<tr>
<td></td>
<td>4 μg/mL</td>
<td>1.086±0.287***</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 versus control group; ***P<0.01 versus model group.

OD: Optical density
and 64.84%, respectively ($P < 0.05, 0.01,$ and $0.01$ vs. model group). The decrease rate was positively correlated with the drug concentration [Figure 2 and Table 5].

**Effect of shikonin on protein kinase C phosphorylation protein content in cytoplasm**

The results displayed that the content of PKC phosphorylation protein in activated T lymphocytes increased significantly, 62.28% higher than that of the control group ($P < 0.01$). After shikonin (1 μg/mL and 2 μg/mL) adding to the cells, their PKC phosphorylation protein content could significantly reduce by 16.82% and 21.4% compared with those in the model group ($P < 0.05$). The content of phosphoprotein in 0.5 μg/mL was decreased by 9.65% compared with that in the model group without significant difference [Table 5].

**Effect of shikonin on the expression of mRNA in nuclear factor of activated T lymphocytes**

In this experiment, the mRNA expression of nuclear factor of activated T lymphocytes (NF-κB) in the model group was significantly increased, over two times higher than that in the control group ($P < 0.01$). These T lymphocytes added with different concentrations of shikonin, the mRNA expression levels significantly decreased, with a decrease of 31.71%, 32.40%, and 47.74% (all $P < 0.01$ vs. model group). The effect was similar in the groups of 0.5 μg/mL and 1 μg/mL, respectively. The inhibitory effect was most obvious in the 2 μg/mL shikonin group, showing a marked trend with a high-concentration drug effect [Table 6]. The results of c-Jun showed that the expression level of c-Jun mRNA in the model group was 52.05% higher than that in the control group ($P < 0.01$). The expression of c-Jun mRNA in the cells added with 0.5–2 μg/mL shikonin was decreased by 12.28%, 18.13%, and 25.73%, respectively (all $P < 0.05$ vs. model group), which was related to the drug concentrations [Table 6].

**Effect of shikonin on the protein expression of nuclear transcription factor c-Jun and NF-κB in T lymphocytes**

The results showed that the level of protein expression in the model group was much higher than that of the control group. Compared with the model group, the protein expression levels of the shikonin (0.5–2 μg/mL) decreased to some extent [Figure 3]. In addition, the expression level of NF-κB protein in the model
group was greatly higher than that in the control group. After adding shikonin at the concentrations of 0.5–2 μg/mL into the cells, the expression of NF-κB proteins was inhibited with most obvious effect on using 1 μg/mL shikonin [Figure 4].

**DISCUSSION**

Liangxue Huoxue Capsule is a hospital preparation of Beijing Hospital of Traditional Chinese Medicine affiliated to Capital Medical University. The preparation was developed on the basis of improved techniques in the treatment of psoriasis commonly used effective prescription Liangxue Jiedu Decoction by famous dermatologists of Zhao Bingnan and Zhang Zhili. The preparation fully embodies TCM’s pattern identification thought of clearing heat and cooling blood, and invigorating blood and resolving toxins on the treatment of psoriasis in blood-heat syndrome, which is an effective prescription for the treatment of psoriasis under the guidance of blood cooling and toxin resolving.

During the decomposed recipe study of Liangxue Huoxue Capsule, we found a cross point between the blood-cooling herbal group and the toxin-resolving herbal group, namely arnebia root (Radix Arnebiae). Arnebia root, a chief herb of Liangxue Huoxue Capsule, originally records in the Shen Nong’s Classic of the Materia Medica as a middle-level herb. Bitter in flavor and cold in property, it has functions of cooling and invigorating blood, resolving toxins, and promoting eruption of papules.[9] Obviously, arnebia root is a representative herb following the treatment principle of cooling blood and resolving toxins for its dual effect. Clinical studies have shown that the total effectiveness rate of psoriasis vulgaris treated with Zicao Decoction in which arnebia root serves as a chief herb was 85.71%.[10] External application of Zicao cream can effectively improve the symptoms of psoriasis lesions such as dryness, desquamation, and itching, with a total effectiveness rate of 75.86%.[11] Experimental studies have demonstrated that shikonin can reduce the epidermal thickness of psoriatic lesions induced by imiquimod in mice, decrease serum levels of IL-17, IL-6, TNF-α, and IL-22,[12] inhibit the excessive proliferation of keratinocytes, and induce apoptosis,[13,14] the maturation and differentiation of dendritic cells, thereby restraining their ability to promote lymphocyte proliferation and improving the degree of skin lesions in psoriasis-like mice.[15,16]

In this study, shikonin was still selected as a research object to observe its effect on T lymphocyte activation and related signal transduction pathways. The results showed that different doses of shikonin (0.5, 1, and 2 μg/mL) all could obviously reduce the proliferation of T lymphocytes and significantly inhibit the expression of cell surface activation molecule CD69 and Th1 cytokines secreted by T cells in dose-dependent manners, indicating a certain immunosuppression.

The activation of T lymphocytes is controlled and regulated strictly. Among them, at the early stage, the activation of PKC and the increase of intracellular Ca²⁺ concentration are the central steps in regulating the activation of T cells. On the one hand, PKC activation can activate the heterodimer Activator protein I (AP-1) developed by the binding of Fos and Jun proteins through classical diacylglycerol (DAG), Rat sarcoma (Ras), Rapidly accelerated fibrosarcoma (Raf) and Mitogen-activated protein kinase kinase kinase (MAPKKK) pathways. On the other hand, signal molecules such as B-cell lymphoma-10 (BCL-10), Carma1, and Mucosa-associated lymphoid tissue MALT-1 can directly activate NF-κB.[17] Constant intracellular Ca²⁺ signaling can induce CaN phosphorylation, leading to dephosphorylation of NF-AT and activation, and translocation into the nucleus.[18] The activated NF-AT binds to AP-1 produced by the PKC pathway to form a transcription complex that binds to regulatory elements of the target gene, which regulate the expression of the
target gene jointly. The activated NF-κB translocates into the nucleus after its dissociation with its inhibitory protein IκBα. There are multiple binding sites for IL-2, IFN-γ, TNF-α, and other genes on its sequence, which can directly regulate a variety of cytokines expression.

The study results of shikonin on T cell activation signal transduction pathway display that shikonin (0.5, 1, and 2 μg/mL) all can significantly reduce the intracellular free Ca\(^{2+}\) concentration and PKC phosphorylation protein content, indicating that shikonin can exert inhibitory effects on the second messenger at upstream of the signal pathway and the key node kinases. This suggests that shikonin may also have a regulatory effect on downstream of the signal transduction pathway, thereby regulating the entire inflammatory response. Based on the expression of nuclear transcription factor NF-AT, AP-1, NF-κB mRNA, and protein at downstream of the signal pathway, the results during experiments confirmed that different doses of shikonin all have significant inhibitory effects on the expression of NF-AT mRNA. The higher the concentration is, the more remarkable the inhibition exerts. Second, each dose of shikonin can effectively reduce the mRNA expression of c-Jun, a major component of AP-1, and great inhibition on its protein expression. Inhibition of c-Jun protein expression can effectively restrain the formation of AP-1 heterodimer, thereby inhibiting the biological activity of AP-1, which is unable to bind with NF-AT in the nucleus, thereby controlling the expression of target gene jointly. In addition, each group of shikonin can inhibit the protein expression of NF-κB, of which the most marked effect is 1 μg/mL shikonin.

In summary, this study demonstrates that shikonin can inhibit the proliferation, activation, and release of cytokines of T lymphocytes and presents with a certain immunosuppressive effect. At the same time, it also proves that shikonin can decrease the concentration of Ca\(^{2+}\) in second messenger by downregulation of signal transduction pathways and the content of PKC in key node kinase and inhibit the mRNA and protein expression of nuclear transcription factors such as NF-AT, AP-1, and NF-κB, thus regulating the signal transduction pathway of T lymphocyte activation. This study provides an experimental basis for the treatment of psoriasis using shikonin as an immunosuppressive agent.

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Conflicts of interest

There are no conflicts of interest.

References