Flavonoids from Scutellaria barbata inhibit activation of tumor-associated macrophages by blocking the Toll-like receptor 4/myeloid differentiation factor 88/nuclear factor-κB signaling pathway

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OBJECTIVE: To determine the efficacy of Scutellaria barbata flavonoids and polysaccharides on Ishikawa endometrial carcinoma cells co-cultured with U937 macrophages.

METHODS: The presence of CD163 and CD206 was determined by flow cytometry. Thiazolyl Blue Tetrazolium Bromide assays were used to assess the proliferation effect of tumor-associated macrophages (TAMs) on Ishikawa cells. The secretion of interleukin (IL)-10 in the co-culture conditioned media was examined using an enzyme-linked immunosorbent assay. The protein expression levels of Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88) and nuclear factor (NF)-κB p65 were detected by Western blot. The mRNA expression levels of TLR4 and MyD88 were analyzed by real-time polymerase chain reaction (PCR). The expression levels of IL-12, IL-1β and tumor necrosis factor-α (TNF-α) were evaluated with real-time PCR.

RESULTS: Compared with the U937 control group, the expression levels of CD163 and CD206 in the TAM group were higher ($P < 0.05$). TAMs co-cultured with Ishikawa cells for 24 or 48 h showed higher proliferation rates ($P < 0.05$). The expression levels of IL-12 decreased than compared with those in the U937 untreated group ($P < 0.05$) and those of the Scutellaria barbata flavonoids group ($P < 0.05$). The expression levels of CD206, CD163, IL-10, IL-1β and TNF-α, NF-κB p65 and TLR4/MyD88 in the TAMs control group were greater than those in the U937 untreated group ($P < 0.05$) and those of the Scutellaria barbata flavonoids group ($P < 0.05$).

CONCLUSION: Scutellaria barbata flavonoids may inhibit TAM activation by blocking the TLR4/MyD88/NF-κB signaling pathway.

Keywords: Endometrial neoplasms; Scutellaria baicalensis; Flavonoids; Toll-like receptor 4; Myeloid differentiation factor 88; Macrophages

INTRODUCTION

Endometrial carcinomas are common malignant epithelial tumors in female genital tract. In a preliminary study, tumor-associated macrophages (TAMs) were found to play an important role in the occurrence and progression of endometrial carcinomas. TAMs can promote endometrial carcinomas and the formation of blood and lymphatic vessels of precancerous tissues via the Toll-like receptor 4/myeloid differentiation primary response 88 (TLR4/MyD88) signaling pathway. In addition, TAMs are associated with poor prognoses.
Traditional Chinese medicine (TCM) has recently been recognized for their advantages in anti-tumor treatments. This study was aimed to examine the effects of flavonoids and polysaccharides extracted from Scutellaria barbata on Ishikawa human endometrial carcinoma cells co-cultured with TAMs and also investigated the molecular mechanism underlying the actions.

**METHODS**

**Cells and treatment**

Ishikawa human endometrial cancer cells were obtained from the Gynecology Laboratory of Peking University People’s Hospital and cultured in DMEM/F12 Cell Medium (Gibco, CA, USA). Human lymphoma macrophage U937 cells were purchased from the Cell Resource Center of Peking Union Medical College Hospital and cultured in RPMI-1640 cell medium (Gibco, CA, USA). Scutellaria barbata flavonoids and polysaccharides were purchased from Nanjing Ze Lang Pharmaceutical Technology, Ltd. (batch No. ZL20131124BH and ZL20131124BD).

**3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide**

Ishikawa cells were digested with D-trypsin and seeded onto 96-well plates (Corning, NY, USA) at a cell inoculum density of 2 × 10^4 cells/mL. After 12 h of incubation, the cells were divided into TAM, U937 control, SBF and Scutellaria barbata polysaccharide (SBP) groups. The four groups were cultured for 24 or 48 h; then 20 μL of MTS (Promega, WI, USA) were added to each culture, which was then incubated for 1 h. The absorption spectra were measured at 490 nm with a plate reader. The tests were performed in duplicate. The inhibition of cell growth was calculated with the formula: cell growth inhibition rate = 1 – (group experimental group OD value – blank OD value)/group (control group OD value- blank OD value).

**Flow cytometry**

The TAM model was established according to the TAM co-culture system. Cells were divided into TAM, U937 control and SBF (400 mg/mL, 24 h) groups. The three groups were cultured for 24 h; then the supernatants were collected. The two cell types were mixed and washed three times with PBS; then 200 μL of a CD163 antibody (1:25) (Abcam, USA) was added to the TAM and SBF groups, and 200 μL of PBS was added to the untreated U937 group. The three groups were incubated for 60 min and washed twice with PBS. Then 200 μL of FITC-labeled antibody (1:50) was added, and the cells were incubated for 15 min in the dark. Finally, the cells were suspended in 200 μL of PBS. The method of detection of CD206 (Abcam, Cambridge, England) expression in cells was similar to that of CD163 detection, except 200 μL of a CD206 antibody (1:50) was added to the three groups, which were then incubated for 15 min.

**Enzyme-linked immunosorbent assay**

After the treatments were applied, the conditioned culture media of the three groups were collected and analyzed for IL-10 expression by enzyme-linked immunosorbent assay (Jingtian Bio, Shanghai, China). Agents A and B were added to untreated wells and terminated liquid. Then 50 μL of standard and 50 μL of streptomycin-HRP were added to the standard wells. Next, 40 μL of sample, 10 μL of antibody and 50 μL of streptomycin-HRP were added to the sample wells. All plates were incubated for 1 h. Following the manufacturer’s instructions, after the stop solution was added, the sample absorptions were measured at 450 nm with a plate reader. The tests were performed in duplicate.

**Western blot**

Cells were lysed in lysis buffer (Ripa, Applygen, Beijing, China) to extract whole proteins. The protein content was determined by a BCA kit (Applygen, Beijing, China). Then the samples were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter membrane (Applygen, Beijing, China). After the membrane was blocked with milk, anti-NF-kB p65 (Abcam, Cambridge, England), anti-TLR4 (Abcam, Cambridge, England) and anti-MyD88 (Abcam, Cambridge, England) and anti-GAPDH (Thermo, MA, USA) antibodies were added.

**Real-time reverse transcription-polymerase chain reaction**

Total RNA was isolated and extracted with TRIzol reagent (Invitrogen, CA, USA). Then the OD values of the total RNA at wavelengths of 260 and 280 nm were detected with an ultraviolet spectrophotometer, and the purity of the RNA was evaluated, followed by real-time RT-PCR amplification using specific primers. GAPDH was used as an internal standard. Primer sets used were as follows: MyD88 5′ GGCTGCTCTCAACATGCGA3′ and 5′ CTGTTGCACGTTCAGA3′ (57°C); TLR-4 5′ AGTTGACCTAAAGCCTGAGT3′ and 5′ GCTGTTGTCCTCAATCATT3′ (57°C); GADPH 5′ CTGGCTACTAGCTGACCC3′ and 5′ CTTGGCTGACCTGACCC3′ (57°C); IL12 5′ AGGGCTGCTACACATG3′ and 5′ TCTGCAAGTGCATAGC3′ (57°C); TNF-α 5′ GGCTGCTGACCTGACCC3′ and 5′ ATCTCTCGCTCCAGCCATT3′ (57°C).
**Statistical analysis**

Data are expressed as means ± standard deviations (x ± s). One-way analysis of variance (ANOVA) was performed to test the differences between groups using SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA). A P < 0.05 was significant level.

**RESULTS**

**Proliferation of Ishikawa cells**

The result revealed that the inhibition rates within a certain range showed a dose-dependent effect (Figure 1A and 1B). The proliferation of Ishikawa cells was observably suppressed for 24 h treatment with Scutellaria barbata flavonoids (98.5%) at a concentration of 800 μg/mL (Figure 1A). However, in cells cultured for 48 h, the inhibition rate reached a maximum and then declined to 96.7% (Figure 1B). When Ishikawa cells were treated with the different concentrations of Scutellaria barbata polysaccharides for 24 and 48 h, the inhibition rates in the Ishikawa cell cultures were less than 15% (Figure 1C and 1D). The inhibition rates of the different concentrations were negative and less variable, and no significant differences were observed.

**Transformation of M1 type to M2 type macrophages**

The expression levels of CD163 and CD206 were detected by flow cytometry. The expression levels of CD163 and CD206 were 3.3% and 70.03% in the TAM control group and 2.1% and 57.2% in the U937 untreated group, respectively. The expression levels of CD163 and CD206 in the TAM control group were higher than those in the U937 untreated group. Results from the Flow cytometry showed that the expression levels of CD206 and CD163 in the SBF group were lower than those in the TAM control group (Figures 2, 3).

Compared with the U937 untreated group, the expression level of IL-10 in the TAM control group increased and the expression level of IL-12 decreased. Further-
more, the expression level of IL-10 in the TAM control group was higher than that in the SBF group and the expression level of IL-12 in the TAM control group was lower than that in the SBF group (Figure 4).

**DISCUSSION**

In the 19th century, Rudolf Virchow found that many malignant tumors were often accompanied by significant and chronic infiltration of inflammatory cells. However, the relationship between inflammation and tumors has only recently attracted attention. Researchers have confirmed that the tumorigenesis of many systemic tumors is related to the inflammatory response and plays an important role in tumor occurrence, development, invasion and the different phases of metastasis. However, it has been confirmed that chronic inflammation plays a crucial role in a variety of malignant tumors. Researchers have shown that the TLR4/MyD88 signal transduction pathway plays an important role in inflammation and tumor development.

Chronic inflammation can develop into breast, liver, gastric and colon cancers and other substantive tumors. Evidence indicates that NF-κB can promote and accelerate the proliferation of tumor cells by continuous activation, cell apoptosis inhibition, increased VEGF levels, promotion of angiogenesis, and induction of the expression of MMP. NF-κB is an important intermediate molecule in inflammation and tumor progression. First, in the inflammatory microenvironment, the TLR4 signaling pathway uses MyD88 as a starting factor. Then the NF-κB signaling pathway activates the release of cytokines and active materials that produce an anti-inflammatory effect. This pathway is called the TLR4/MyD88-NF-κB signaling pathway. Researchers have found that endometrial carcinomas are responsible for significant TAM infiltration and associated with a poor prognosis in patients. While the anti-tumor effects of TCM have recently been revealed, the targets and anti-tumor mechanisms of TCM on the TAM effective “groups” are unknown. Furthermore, the role of TAMs in the occurrence and progression of endometrial carcinoma is not clear. Recent studies have investigated the regulation of inflammation by the TLR4/MyD88 signaling transduction pathway in estrogen-receptor-positive breast, prostate and ovarian cancers. However, few studies have explored the correlation between endometrial ade-
These chemicals and detoxifying TCM had anti-tumor proliferation, and exacerbate endometrial lesions. Conversely, M1 signaling pathways can block the expression of this pathway and thereby inhibit the differentiation and activation of TAMs. Scutellaria barbata flavonoids can down-regulate the level of CD163, CD206 and IL-10, and CD163 and CD206 themselves are involved in the regulation of IL-10. We assumed that Scutellaria barbata flavonoids act by lowering the expression level of IL-10 to reverse the M1 to M2 macrophage transformation.

Another feature of macrophage activation is the activation of the TLR4/MyD88-NF-κB signaling pathway. NF-κB often binds to other molecules via its p50 and p65 subunits. This signaling pathway plays a key role in the differentiation and activation of TAMs. The inhibition of TLR4/MyD88-NF-κB can cause the phenotype of TAMs to change from M2 to M1. This study confirmed that the expression of the NF-κB p65 subunit in TAMs was higher than in U937 cells. It can be shown that the TLR4/MyD88-NF-κB signal transduction pathway plays a role in the differentiation and activation of TAMs. Scutellaria barbata flavonoids can block the expression of this pathway and thereby inhibit the M1 to M2 macrophage transformation in the tumor microenvironment.

In conclusion, Scutellaria barbata flavonoids can re-

Figure 5 Expression levels of TLR4/MyD88-NF-κB in three groups after 24 h with Scutellaria barbata flavonoids (800 μg/mL)

Figure 6 Expression levels of IL-1β and TNF-α in three groups after 24 h with Scutellaria barbata flavonoids (800 μg/mL)

nocarcinomas and the signaling pathways and have only examined these relationships in bovine endometrial cells. To our knowledge, no studies have investigated TLR4/MyD88 signaling pathway. Our previous study showed that the TLR4/MyD88 and NF-κB signal transduction pathways play key roles in the differentiation and activation of TAMs. The inhibition of the TLR4/MyD88 signaling pathways can cause TAMs to change from the M2 to M1 phenotypes. Conversely, M2 type macrophages can express a high level of CD206 and IL-10, promote Ishikawa cell proliferation, and exacerbate endometrial lesions. In preliminary studies, we found that berberine and matrine can prevent TAMs from promoting Ishikawa cell proliferation. In a nude mouse in vitro tumor transplantation experiment, we found that replenishing these chemicals and detoxifying TCM had anti-tumor effects. Scutellaria barbata is mainly used for the treatment of digestive system tumors. In recent years, it has also been used to combat lung cancers, nasopharyngeal carcinomas and other systemic tumors. Although the anti-tumor function of Scutellaria barbata is commonly accepted, the specific anti-tumor effects of its constituent compounds are not clear. At present, the anti-tumor agents from Scutellaria barbata are believed to be flavonoids and polysaccharides. However, the inhibition of cancer or endometrial cell proliferation by these compounds has not been reported. In this study, we detected the expression of the phenotype biomarkers of TAMs (i.e., CD163, CD206 and IL-10) in the supernatant to verify the induction of TAM expression in the Ishikawa-U937 co-cultures. Macrophages in the tumor microenvironment were detected as M2 macrophages, and the expression of IL-10 increased. Scutellaria barbata flavonoids can down-regulate the level of CD163, CD206 and IL-10, and CD163 and CD206 themselves are involved in the regulation of IL-10. We assumed that Scutellaria barbata flavonoids act by lowering the expression level of IL-10 to reverse the M1 to M2 macrophage transformation.
duce the expressions of IL-10 and NF-κB p65 and regulate the TLR4/MyD88 pathway in Ishikawa-TAM co-cultures to prevent the transformation of M1 type to M2 type macrophages. Furthermore, by inhibiting the TLR4/MyD88-NF-κB signal transduction pathway and reducing the expression of IL-10, the activation and transformation of TAMs may be reversed and may elicit an anti-tumor effect in Ishikawa cells. Blocking this signal transduction pathway may be a new method for the management of endometrial cancer.

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REFERENCES