Proceedings of Anticancer Research, 2022, Volume 6, Issue 5
http://ojs.bbwpublisher.com/index.php/PAR
ISSN Online: 2208-3553
ISSN Print: 2208-3545

Mechanism of Ginsenoside Rh1 in Regulating Breast Cancer Cell Extravasation Based on CCL20-CCR6

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Abstract: *Objective:* To explore the regulatory mechanism of ginsenoside Rh1 on breast cancer cell extravasation based on CCL20-CCR6. *Methods:* In 2021, a total of 34 patients with breast cancer were treated in Baoding First Central Hospital. During hospitalization, pathological examinations were performed, and all the patients were diagnosed with invasive ductal carcinoma. Out of the 34 cases, 16 cases were found to have CCR6 expression in breast cancer tissues, in which they were recorded as the CCR6 expression group, whereas 18 cases did not have CCR6 expression; these cases were recorded as the CCR6 non-expression group. During the same period, 21 normal patients were selected as the control group. The peripheral blood CCL20 level and the expression of CCR6 on the surface of $CD3^+$ T lymphocytes were analyzed. The extracts of cancer cells were collected, purified, and cultured, and the effect of ginsenoside Rh1 on the invasion and metastasis of breast cancer cells was analyzed. *Results:* The peripheral blood CCL20 level in the CCR6 expression group was significantly higher than that in the CCR6 non-expression group and the control group, in which $p < 0.05$, indicating that the difference was statistically significant; at 12, 24, and 48 hours, the cell survival rate of each dose group was significantly higher than that of the blank control group and the dimethyl sulfoxide (DMSO) group ($p < 0.05$). At 48 hours, comparing the low-dose group with the high-dose group, the cell survival rate significantly decreased ($p < 0.05$). Compared with the blank control group and DMSO group, the invasion ability of breast cancer cells could be reduced in both, high- and medium-dose groups, where $p < 0.05$, indicating that the difference was statistically significant. *Conclusion:* CCL20 may play a role in the pathogenesis of certain breast cancers, and ginsenoside Rh1 can effectively regulate the invasion and migration of breast cancer cells.

Keywords: CCL20; CCR6; Ginsenoside Rh1; Breast cancer

Online publication: September 28, 2022

1. Introduction

Breast cancer is the most commonly diagnosed malignancy in women worldwide. Although resection has improved the survival rate of breast cancer to a certain extent, short-term recurrence or metastasis may still occur to many patients who had received chemotherapy due to the resistance of cancer cells during later chemotherapy. Therefore, research is focused on discovering new and effective therapeutic targets. The breast tumor microenvironment (TME), consisting of recruited host stromal cells and tumor cells, has recently emerged as an important player in tumor progression with therapeutic potential. TME contains components of the immune system, such as macrophages and lymphocytes, cells that make up the blood vessels, mesenchymal stem cells, and extracellular matrix (ECM). Among these components, tumor-associated macrophages (TAMs) are important components of the TME in breast cancer. Macrophages are

involved in innate and adaptive immune responses to control multiple TME factors. In human breast cancer, high TAM density is associated with poor prognosis. Over the years, research into the role of TAMs in breast cancer progression has established that TAMs are capable of inducing angiogenesis, remodeling the tumor extracellular matrix to aid invasion, modeling breast cancer cells to evade the host immune system, and depleting immunosuppressive leukocytes recruited into the tumor microenvironment [1–7]. Therefore, elucidating which signaling pathways regulate the development, differentiation, activation, inactivation, and apoptosis of TAM during the contact between macrophages and tumors would provide new strategies for targeting the inhibition of breast cancer growth and insinuating potential targets for therapeutic intervention.

2. Materials and methods

2.1. Experimental subjects and materials

(1) In 2021, a total of 34 patients with breast cancer were admitted to Baoding First Central Hospital. Pathological examinations were performed, and all the patients were diagnosed with invasive ductal carcinoma. Among these 34 patients, 16 cases were found to have CCR6 expression in breast cancer tissues, in which the cases were recorded as CCR6 expression group, whereas 18 cases did not have CCR6 expression, in which the cases were recorded as CCR6 non-expression group. During the same period, 21 normal patients were selected as the control group. The peripheral blood CCL20 level and the expression of CCR6 on the surface of $CD3^+$ T lymphocytes were analyzed.

(2) Triton X-100, FITC-Labeled Goat Anti-Rabbit IgG (H+L) Kit, fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), trypsin-EDTA solution, ginsenoside Rh1, and dimethyl sulfoxide were used. According to the experimental requirements, the ginsenoside Rh1 proportion of the drug was divided into the low-dose group: 250 μmol/L; middle-dose group: 500 μmol/L; and high-dose group: 750 μmol/L.

2.2. Methods

(1) On an empty stomach, 3 mL of blood was taken from the cubital vein and centrifuged at 3,000 r/min (centrifugation radius 15 cm) for 10 minutes, in which the serum was separated for use. 1 mL of anticoagulant was used for the detection of CCR6. The test steps were carried out in strict accordance with the Chemokine CCL20 Detection Kit (RD Company, United States) instructions; the whole blood two-color fluorescent labeling method was used for detecting the chemokine receptor CCR6 on the surface of peripheral blood $CD3^+$ T lymphocytes.

(2) The cancer cell extracts from the postoperative breast cancer tissues of the patients were purified and cultured; single cell suspension was prepared and plated in a 96-well plate, and the number of cells was adjusted. The initial cell number for proliferation studies was 2×10^5 cells per well. After gently mixing, the cells were placed in a cell incubator for 16 hours, and the drugs were added when the confluence rate reached 80%. The blank control group, DMSO group, and ginsenoside Rh1 low-, medium-, and high-dose groups were set up, and 4 to 6 replicates were set for each group. The drugs were diluted with culture medium and then added. 100 μL from each well was incubated in 5% carbon dioxide (CO_2) at 37°C, standing at room temperature to dissolve MTS; 20 μL Cell Titer96® AQueous One Solution Reagent was added in 100 μL medium with 8 tubes. After incubation with 5% CO_2 at 37°C for 2 hours, the absorbance value was read at 490 nm at 3 time points: 0, 24, and 48 hours. Breast cancer cell lines were detected by flow cytometry, reverse transcription-polymerization chain reaction (RT-PCR), western blot, and Transwell experiments.

2.3. Observation indicators

The levels of CCL20 in peripheral blood, the expression of CCR6 on the surface of $CD3^+$ T lymphocytes,

and the effect of ginsenoside Rh1 on the invasion and metastasis of breast cancer cells were observed and analyzed.

2.4. Statistical analysis

Statistical Package for the Social Sciences **(**SPSS) 18.0 was used for statistical analysis of experimental data. All experiments were independently repeated at least three times. The measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± s), and t-test was used for data comparison and analysis. $p < 0.05$ indicated statistical significance.

3. Results

3.1. Analysis of CCL20 levels in peripheral blood and expression of CCR6 on $CD3^+$ T lymphocytes

The peripheral blood CCL20 level in the CCR6 expression group was significantly higher than that in the CCR6 non-expression group and the control group, in which $p < 0.05$, indicating that the difference was statistically significant, as shown in **Table 1**.

Table 1. Analysis of CCL20 levels in peripheral blood and expression of CCR6 on $CD3^+$ T lymphocytes

Group	Number of cases	CCL20 (ng/mL)	CCR6 (%)
CCR6 expression group	16	24.78 ± 19.12[a,b]	2.45 ± 2.14
CCR6 non-expression group	18	6.41 ± 4.22	2.18 ± 1.98
Control group	20	5.78 ± 4.12	2.41 ± 1.78

Note: [a] means compared with the CCR6 non-expression group, $p < 0.05$; [b] means compared with the control group, $p < 0.05$

3.2. Analysis of the inhibitory effect of ginsenoside Rh1 on the proliferation of breast cancer cells

At 12, 24, and 48 hours, the cell viability in each dose group was significantly lower than that in the blank control group and the DMSO group ($p < 0.05$). At 48 hours, the cell viability in the high- and medium-dose groups significantly decreased compared to the low-dose group ($p < 0.05$) as shown in **Table 2**.

Table 2. Analysis of the inhibitory effect of ginsenoside Rh1 on the proliferation of breast cancer cells

Group	Cell viability (%)			
	0 hour	12 hours	24 hours	48 hours
Blank control group	101.11 ± 7.41	100.69 ±7.41	99.45 ± 6.87	98.12 ± 4.58
DMSO group	100.41 ± 6.98	98.89 ± 6.78	97.89 ± 6.98	97.14 ± 4.89
Low-dose group	97.89 ± 6.78	82.69 ± 5.89	71.36 ± 6.98	68.78 ± 4.25
Medium-dose group	99.12 ± 6.89	78.69 ± 6.97	61.26 ± 6.45	48.89 ± 5.24
High-dose group	100.12 ± 7.04	76.78 ± 6.48	60.47 ± 6.35	43.69 ± 5.78

Note: After 48 hours, the middle- and high-dose groups were compared with the blank control, DMSO, and low-dose groups, respectively, in which all comparisons showed $p < 0.05$

3.3. Transwell assay to detect ginsenoside Rh1 inhibits the invasion of breast cancer cells

Compared with the blank control group and DMSO group, the invasive ability of breast cancer cells could be reduced in both the high- and medium-dose groups, where $p < 0.05$, indicating that the difference was statistically significant, as shown in **Table 3**.

Table 3. Transwell assay to detect the ability of ginsenoside Rh1 to inhibit the invasion of breast cancer cells

Group	Number of cells
Blank control group	88.12 ± 7.01
DMSO group	91.24 ± 7.21
Low-dose group	84.78 ± 5.96
Medium-dose group	57.12 ± 3.48
High-dose group	50.47 ± 4.12

Note: The middle- and high-dose groups were compared with the blank control, DMSO, and low-dose groups, respectively, in which all comparisons showed $p < 0.05$

4. Discussion

4.1. Breast cancer and CCL20-CCR6

Many cytokines play specific roles in the occurrence and development of breast cancer. They influence the growth of breast cancer by regulating the self-renewal of breast cancer stem cells (BCSC). Some cytokines regulate the progression of cancer by regulating the microenvironment of breast cancer. In patients with breast cancer, the overall survival and metastasis-free survival decrease with high CCL20 expression. Intraperitoneal injection of anti-CCL20 antibody can inhibit osteolytic bone metastasis of breast cancer cells in mice [8,9]. CCL20 treatment significantly promotes the invasion and matrix metalloproteinase (MMP)-2/9 secretion of basal-like triple-negative breast cancer (TNBC) cells, further confirming the role of CCL20 in bone metastasis of breast cancer. In a xenograft model, the overexpression of CCL20 has been found to promote the proliferation and invasiveness of TNBC cells and accelerate tumor growth. Moreover, CCL20 promotes the self-renewal of breast cancer stem cells and enhances the expansion capacity of the cancer stem cell population. This suggests that CCL20 can regulate breast cancer growth by affecting cancer stem cells. CCL20 can also enhance the chemoresistance of breast cancer cells, activate the NF-κB pathway through protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK), promote the expression of the protein ABCB1 responsible for drug efflux, and subsequently allow taxanes to be continuously pumped out of cells, thereby enhancing cancer drug resistance in cells. CCL20 may promote cell invasion by activating PKCα of Src, which may also lead to the activation of downstream Akt, JNK, and NF-kB pathways. In addition, CCL20 regulates epithelial-mesenchymal transition (EMT) in predominantly cultured healthy mammary epithelial cells adjacent to tumor areas by downregulating E-cadherin and ZO-1 as well as upregulating the expression of N-cadherin, Vimentin, and Snail [10–12]. Therefore, targeting CCL20 or downstream pathways activated by CCL20 (such as NF-κB) can significantly improve the treatment outcomes of patients with breast cancer, especially those with triple-negative breast cancer. Breast cancer cells in primary cultures of breast cells taken from normal peritumoral areas express CCR6, which activates various signaling kinases involved in CCL20-induced proliferation and migration of breast cells. The above evidence suggests that the occurrence and development of breast cancer is closely related to CCL20-CCR6, but the specific mechanism needs to be further explored.

4.2. CCL20-CCR6 and macrophages

Among various immune factors involved in the mechanism of TAM-induced cancer cell migration, chemokines have been reported as key factors promoting cancer migration. Culture media (CM) human cytokine antibody arrays co-cultured with Caki-1 cells (human renal cancer cell line) with different states of THP-1 cells (human leukemia monocytic cell line) showed higher CCL20 concentrations in CMs co-cultured with macrophage-like cells. More importantly, CCL20 alone has been found to increase chemokine

secretion between M1L and M2L macrophages in CMs co-cultured with macrophage-like cells. This difference in CCL20 secretion is proportional to the migratory effect of macrophage-like cells on renal cell carcinoma (RCC) cells, and CCL20 can be regarded as a key chemokine that directly regulates RCC cell behavior and promotes CC cell migration when the CCL20-CCR6 axis is inhibited. Multiple studies have revealed the role of CCL20 and its specific receptor CCR6. Data have suggested that TAMs may secrete CCL20, while CCR6-expressing RCC cells deplete CCL20 in the tumor microenvironment, resulting in enhanced RCC cell migration capacity. Studies have shown that the CCL20-CCR6 axis in macrophage-RCC cell interaction can induce Akt activation and EMT in RCC cells. The activation of Akt-induced EMT changes via the CCL20-CCR6 axis has also been reported in gastric and cervical cancer cells. Moreover, the RCC tissue specimens revealed that the high expression of CCR6 levels were not only associated with advanced states of RCC, but also significantly shortened the overall survival of RCC patients. Therefore, the CCL20-CCR6 axis may be a novel biomarker and therapeutic target for tumors. However, there are still some issues that need to be resolved and are worth exploring. No study has yet evaluated the role of CCL20-CCR6 in breast cancer macrophages. Additional clinical evidence is needed to confirm CCL20-CCR6 axis as a biomarker for breast cancer.

4.3. Ginsenoside Rh1 and breast cancer

The main active ingredients of ginseng are ginsenosides. Ginsenosides are mainly divided into three categories: oleanolic acid (OA), protopanaxadiol (PPD), and protopanaxatriol (PPT), of which ginsenoside Rh1 belongs to the PPT category. [13]. With extensive pharmacological research on ginseng, studies have confirmed that ginsenoside Rh1, as one of the active components of ginseng, not only regulates the functions of the central nervous system, cardiovascular system, cerebrovascular system, immune system, *etc*., but also plays an important role in tumor [14]. Ginsenoside Rh1 exists as free or synthetic glycosides in ginseng, and scholars have pointed out that it has significant inhibitory effect on the invasion and metastasis of human hepatoma cells and malignant glioma cells [15,16].

In conclusion, CCL20 may play a role in the pathogenesis of certain breast cancers, and ginsenoside Rh1 can effectively regulate the invasion and migration of breast cancer cells.

Funding

This work was supported by grants from The Medical Science Research Project of Hebei Province (20220282), the Key Laboratory of Molecular Pathology and Early Diagnosis of Tumor in Hebei Province.

Disclosure statement

The authors declare no conflict of interest.

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