

Berberine suppresses migration of MCF-7 breast cancer cells through down-regulation of chemokine receptors

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ARTICLE INFO

Article type:

Original article

Article history:

Received: May 5, 2015

Accepted: Oct 8, 2015

Keywords:

Anticancer agents
Breast cancer
Berberine
Chemokine receptors
Metastasis

ABSTRACT

Objective(s): Berberine is one of the main alkaloids and it has been proven to have different pharmacological effects including inhibition of cell cycle and progression of apoptosis in various cancerous cells; however, its effects on cancer metastasis are not well known. Cancer cells obtain the ability to change their chemokine system and convert into metastatic cells. In this study, we examined the effect of berberine on breast cancer cell migration and its probable interaction with the chemokine system in cancer cells.

Materials and Methods: The MCF-7 breast cancer cell line was cultured, and then, treated with berberine (10, 20, 40 and 80 µg/ml) for 24 hr. MTT assay was used in order to determine the cytotoxic effect of berberine on MCF-7 breast cancer cells. Wound healing assay was applied to determine the inhibitory effect of berberine on cell migration. Moreover, real-time quantitative PCR analysis of selected chemokine receptors was performed to determine the probable molecular mechanism underlying the effect of berberine on breast cancer cell migration.

Results: The results of wound healing assay revealed that berberine decreases cell migration. Moreover, we found that the mRNA levels of some chemokine receptors were reduced after berberine treatment, and this may be the underlying mechanism for decreased cell migration.

Conclusion: Our results indicate that berberine might be a potential preventive biofactor for human breast cancer metastasis by targeting chemokine receptor genes.

► *Please cite this article as:* Ahmadiankia N, Kalalian Moghaddam H, Mishan MA, Bahrami AR, Naderi-Meshkin H, Bidkhorri HR, Moghaddam M, Aldin Mirfeyzi SJ. Berberine suppresses migration of MCF-7 breast cancer cells through down-regulation of chemokine receptors. Iran J Basic Med Sci 2016; 19:125-131.

Introduction

Breast cancer is the most common cancer among women worldwide and the second leading cause of cancer-related mortality in developed countries. Most breast cancer-related deaths are not the result of primary tumor growth but are rather caused by the metastasis of cancer to other organs (1). Metastasis is the last stage in the progression of cancer during which cancer cells detach from the primary tumor, migrate into blood vessels, disseminate throughout the body, and ultimately seeding in distant organs and give rise to new tumors. Several studies have shown that chemokines and chemokine receptors play a key role in cancer metastasis. Chemokines are expressed in specific organs and their interactions with the correspondent receptors which are expressed on tumor cells induce

directed cell migration (2). The extent of this response triggered by chemokines depends on the amount of chemokine receptors expressed on the plasma membrane (3). Chemokine receptors may potentially facilitate tumor dissemination via activation of key survival pathways such as MEK1/2 and PI3/AKT (4). Chemokine receptors belong to a subfamily of G protein-coupled receptors and they are named based on the chemokine ligands to which they bind. So, they are classified into four groups of (I) CXC receptors (CXCR1, 2, 3, 4 and 5), (II) CC receptors (CCR1, 2, 3, 4, 5, 6, 7, 8, and 9); (III) CX3C and (IV) the receptor of XC. It has been suggested that interactions of some specific chemokine receptors with their ligands play important roles in many of the critical steps of the metastatic process (5). Among different classes of chemokine receptors,

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CXCR4 was found to be the most common chemokine receptor overexpressed in different human malignancies including breast cancer. It was revealed that CXCR4 and its ligand (CXCL12) have a key role in initiating and regulating tumor cell migration and metastasis (6). Moreover, it was revealed that the chemokine receptors CCR1, CCR6, CCR9, and CXCR1 are often overexpressed in cancer cells and are involved in cancer metastasis (7-10). Regarding the important role of chemokine receptors in cancer metastasis, scientists focus on developing new drugs to target them. Berberine is an isoquinoline derivative alkaloid with antioxidant and anti-cancer activities. Although pharmacological effects of berberine have been previously reported, recently, special attention was paid to its bioactivities including antioxidant, anti-inflammatory anti-diabetic, neuroprotective effects and its ability as a natural drug for breast cancer treatment (11). Moreover, it was shown that berberine is effective in inhibition of cell proliferation and promotion of apoptosis in different cancerous cells (12). Unlike anti-cancer growth effects of berberine, very little is known about the effect of berberine on cancer cell migration and metastasis. Therefore, in this study, we examined the effect of berberine on migration and invasion of breast cancer cells. Moreover, to clarify the underlying mechanism of berberine effect on migration of breast cancer cells, the expression levels of some selected chemokine genes were analyzed by qPCR. Specific chemokine receptors namely, CCR1, CCR6, CCR9, CXCR1, and CXCR4 were selected since they were previously reported to be associated with cancer metastasis (8). The present study aimed to demonstrate berberine effects against the metastasis of breast cancer cells.

Materials and Methods

Cell culture

MCF-7 human breast cancer cell line was cultured

in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (1% penicillin- streptomycin 10000 units/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. All cell culture reagents were purchased from Sigma.

Cell viability assay

MTT assay was performed to evaluate the cytotoxic effect of berberine on MCF-7 breast cancer cells (7). Briefly, the cells were seeded at a density of 1.2×10^4 cells/ml and incubated with berberine (Sigma) at 0, 10, 20, 40, 80 and 160 μ M for 24 hr. Thereafter, the medium was changed and cells were incubated with MTT (Sigma) solution (0.5 mg/ml) for 4 hr. The number of viable cells is directly proportional to the production of a purple dye, after solubilization with isopropanol which can be measured spectrophotometrically ($\lambda = 545$ nm). The percentage of the viable cells was calculated using the following equation: (mean OD of treated cells/mean OD of control cells) \times 100. Control groups were treated with media containing 0.1% DMSO in all experiments.

Wound-healing Assay

For the wound-healing migration assay, cells were seeded in 6-well plates (8). When cells were confluent, scratch was made with a 200 μ l sterile pipette tip, generating a cell-free area of approximately 1 mm in width. Cellular debris removal was done by gentle washing with culture medium and the photos of the wounds (0 hr) were taken. Thereafter, medium was replaced by culture medium with different concentrations of berberine (0, 10, 20, 40, and 80 μ M) and the cells were allowed to migrate for 24 hr. At the end of migration experiment, another set of photos was taken, from the same regions. The gap size was analyzed using Image-J 1.45 software. In order to assess migration ability of cultured cells, cell-free areas of

Table 1. Description of the designed primers

Gene		Primer sequence	Product size
β -actin	Sense	5'-GCTCAGGAGGAGCAAT-3'	187
	Antisense	5'-GGCATCCACGAAACTAC-3'	
CCR1	Sense	5'-CACGGACAAAAGTCCCTGG-3'	134
	Antisense	5'-CAAAGGCCCTCTCGTTCAC-3'	
CCR6	Sense	5'-GCTAGTGTTCCTCTCATTCC-3'	132
	Antisense	5'-ACCTGTTCTGCCATTGTCC-3'	
CCR9	Sense	5'-TGATTGGCTCTTGACTGTGATG-3'	108
	Antisense	5'-GAGGCAGCGGCATTGATG-3'	
CXCR1	Sense	5'-CATCAGTGTGACCGTTACC-3'	120
	Antisense	5'-GGCAGGGACAGATTCATAGAC-3'	
CXCR4	Sense	5'-ATCCCTGCCCTCCTGCTACTATTG-3'	232
	Antisense	5'-GAGGGCCTTGCGCTTCTGGTG-3'	

the scratches at 24 hr post-wounding were subtracted from the area of the scratches (0 hr) and calculated as a percentage of untreated (0 hr) cultures. The experiment was carried out in triplicates.

RNA extraction and real-time PCR

Total RNAs were isolated from cells using TriPure isolation reagent (Roche, Germany) based on the manufacturer's instructions. Extracted RNA was treated with DNase I enzyme. One microgram of RNA from each sample was used for cDNA synthesis by reverse transcriptase. RNA samples were incubated at 65°C for 5 min with 1 µl OligodT (0.5 µg/µl) at a final volume of 12 µl, and then, chilled on ice and mixed with 4 µl 5X buffer, 2 µl dNTPs (10 mM), 0.5 µl Ribolock, and 1 µl M-MLV-RTase. After incubation at 42°C for 60 min, and at 70°C for 10 min, the mixture was transcribed into cDNA. Synthesized cDNAs were diluted at 1:4 ratio and 2 µl cDNA of each sample was used for real-time PCR in a 20 µl reaction mixture with 10 µl of 2X SYBR Green PCR Mastermix (Parstous, Iran) and 1 µl of specific primer pair. The housekeeping gene, β -actin was used to normalize target gene expression. The primer sequences are presented in Table 1.

Each plate was run at 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. All real-time RT-PCRs were performed in duplicate, and relative mRNA of each target gene was determined by using the formula $2^{-\Delta CT}$ (CT, cycle threshold) where $\Delta CT = CT$ (target gene) – CT (β -actin). The comparative expression level of each target gene between different samples was calculated by $2^{-\Delta\Delta CT}$. In addition, melting curves were used to determine non-specific amplification.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism, version 5.0. The significance of difference among the experimental groups and controls was assessed by one-way ANOVA and *Post hoc* Tukey test. The results are presented as mean \pm standard deviation (SD). $P < 0.05$ represented significant differences.

Results

Berberine decreased the proliferation of human breast cancer cells

In this study, using MTT assay, we determined the cytotoxicity of berberine by treating MCF-7 cells with various concentrations berberine for 24 hr. The results from MTT assay revealed that growth retardation was correlated with increased concentrations of berberine in a concentration-dependent manner (Figure 1). Cell viability was 99%, 89%, 81%, 79%, and 64% for 10, 20, 40, 80, and 160 µM berberine, respectively.

Berberine reduced wound-healing migration of breast cancer cells

Here, we checked whether berberine inhibits cell migration in breast cancer cells. For this purpose, MCF-7 breast cancer cells were subjected to the cell migration assay after 24 hr-treatment with various concentrations of berberine (0, 10, 20, 40 and 80 µM), which were relatively non-toxic for the cells (Figure 2A). Treatment of the cells with berberine resulted in a concentration-dependent reduction in the cell migration capacity of MCF-7 cells as compared to control (Figure 2B).

Berberine decreased the expression of chemokine receptors in breast cancer cells

After observing the decrease in cell migration, we were interested to investigate whether alternations of chemokine receptors expression at mRNA level occurred in MCF-7 cells following berberine treatment. Therefore, qPCR was performed to determine the expression of selected chemokine receptor genes. As shown in Figure 3, 24 hr-incubation with berberine caused no significant effect on the expression of CCR1(A); however, CCR6 was significantly down-regulated following treatment with berberine 40 µM (B). A similar effect was also observed for CCR9 gene expression following treatment with all concentrations of berberine except 10 µM (Figure 3C). Moreover, CXCR1 and CXCR4 were significantly down-regulated following treatment with all concentrations of berberine (Figures 3D and E). These data suggested that the inhibition of chemokine receptors expression in the presence of berberine may resulted in inhibition of MCF-7 breast cancer cell migration.

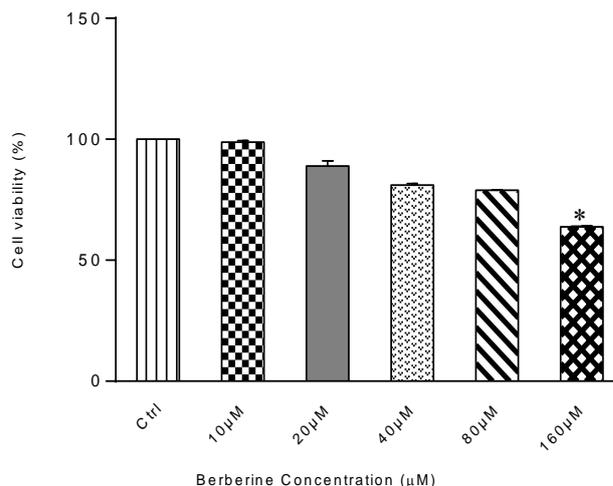


Figure 1. Viable MCF-7 cells after 24 hr-exposure to various concentrations of berberine. Results are represented as mean \pm SD; n=3, (* $P < 0.05$ compared to control)

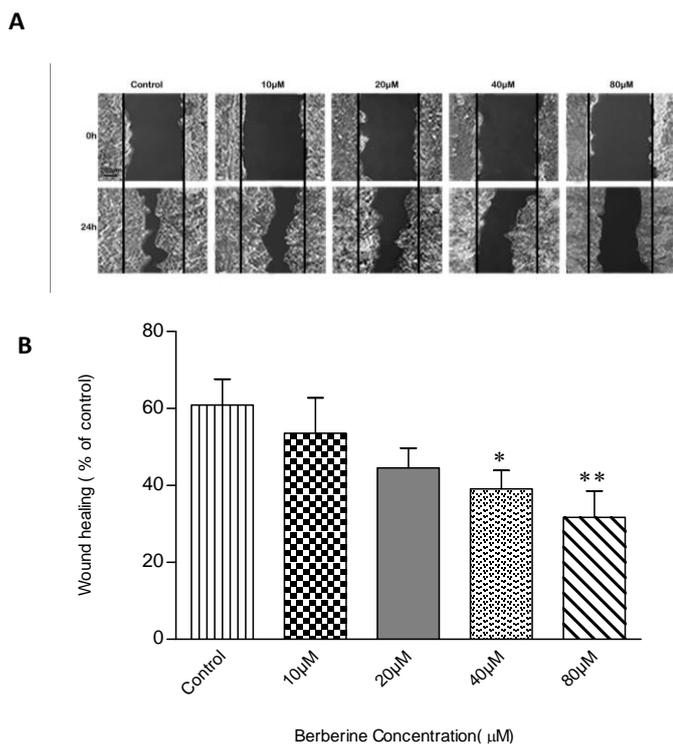


Figure 2. Effect of different concentrations of berberine on cell migration as examined by wound-healing assay. (A) MCF-7 cells grew to 90% confluent and were scratched by a sterile 200 µl pipette tip. The ability of cells to migrate into the scratch area was monitored by photographing the same spot with an inverted microscope equipped with a digital camera, 0 and 24 hr after scratch. Wound-healing images show the extent of healing and black lines indicate the wound edge. Scale bar: 200 µm. (B) Statistical analysis of the migration image results. The graph represents the mean \pm SD for at least three independent experiments. * and ** donate $P < 0.05$ and $P < 0.01$, respectively, as compared to the control

Discussion

The anti-cancer effects of berberine against many different types of human cancers have been well documented (13). In this study, our results revealed that berberine decreased the percentage of viable MCF-7 breast cancer cells in a concentration-dependent manner which is in agreement with previous studies (14). However, there was no available information regarding the effect of berberine on migration and invasion of MCF-7 breast cancer cells and associated signaling pathways. In this study, the results of wound-healing assay indicated that berberine decreased the migration and invasion of viable MCF-7 breast cancer cells and these effects were concentration-dependent. Cancer cells motility and metastasis are multifactorial processes involving different mechanisms (15). Chemokine receptors play an important role in the induction of directed cell migration (3). Therefore, to discover underlying mechanisms involved in

decreased cell migration in response to berberine, we also examined the level of chemokine receptors gene expression after berberine treatment in breast cancer cells.

CC chemokine receptor 1 (CCR1) plays a critical role in the recruitment of leukocytes to the site of inflammation (16). Knockdown of CCR1 resulted in a significantly reduced invasive ability of hepatocellular carcinoma cells and human non-small cell lung cancer cell (7). The potential role of CCR1 in breast cancer cells metastasis has not been studied yet. The result of our study indicated that berberine did not change the expression level of CCR1 in breast cancer cells.

CCR6, another chemokine receptor is closely related to disease stages when up-regulated and its down-regulation has been proposed as one of the strategies to stop metastasis (8). According to the results of our study, CCR6 showed a statistically significant down-regulation when breast cancer cells were treated with berberine 40 µM.

CCR9, another chemokine receptor associated with invasiveness and metastasis of tumors (9) impaired the migration and invasive potential of prostate cancer cells when its expression was blocked, indicating that these responses were dependent on chemokine and chemokine-receptor interactions (17). To our knowledge, for the first time, we demonstrated decreased expression of CCR9 in breast cancer cells treated with berberine. Decreased expression of CCR9 may be the answer to why migration potential of breast cancer cells decreased after berberine treatment.

Among the genes over-expressed in the breast cancer stem cell (CSCs) population, CXCR1, a receptor that binds to the pro-inflammatory chemokines of IL-8/CXCL8 and CXCL6, appeared to have an important role in growth and metastasis of breast CSCs (18). It was shown that CXCR1 blockade resulted in apoptotic induction, retarded tumor growth and reduced systemic metastasis of breast cancer in NOD/SCID mice (10). Human colon cancer liver metastases can be inhibited by small molecule antagonists of CXCR1 (19). Using wound-healing assay, we demonstrated that treatment with berberine resulted in decreased cell migration in breast cancer cells. We also demonstrated a decrease in the expression of CXCR1 in response to berberine. Consistent with these findings, we suggested that berberine may affect migration of breast cancer cells through down-regulation of CXCR1.

CXCR4 is the most common chemokine receptor expressed in tumor cells, and plays a vital role in the migration, invasion, and prognosis of breast cancer (20).

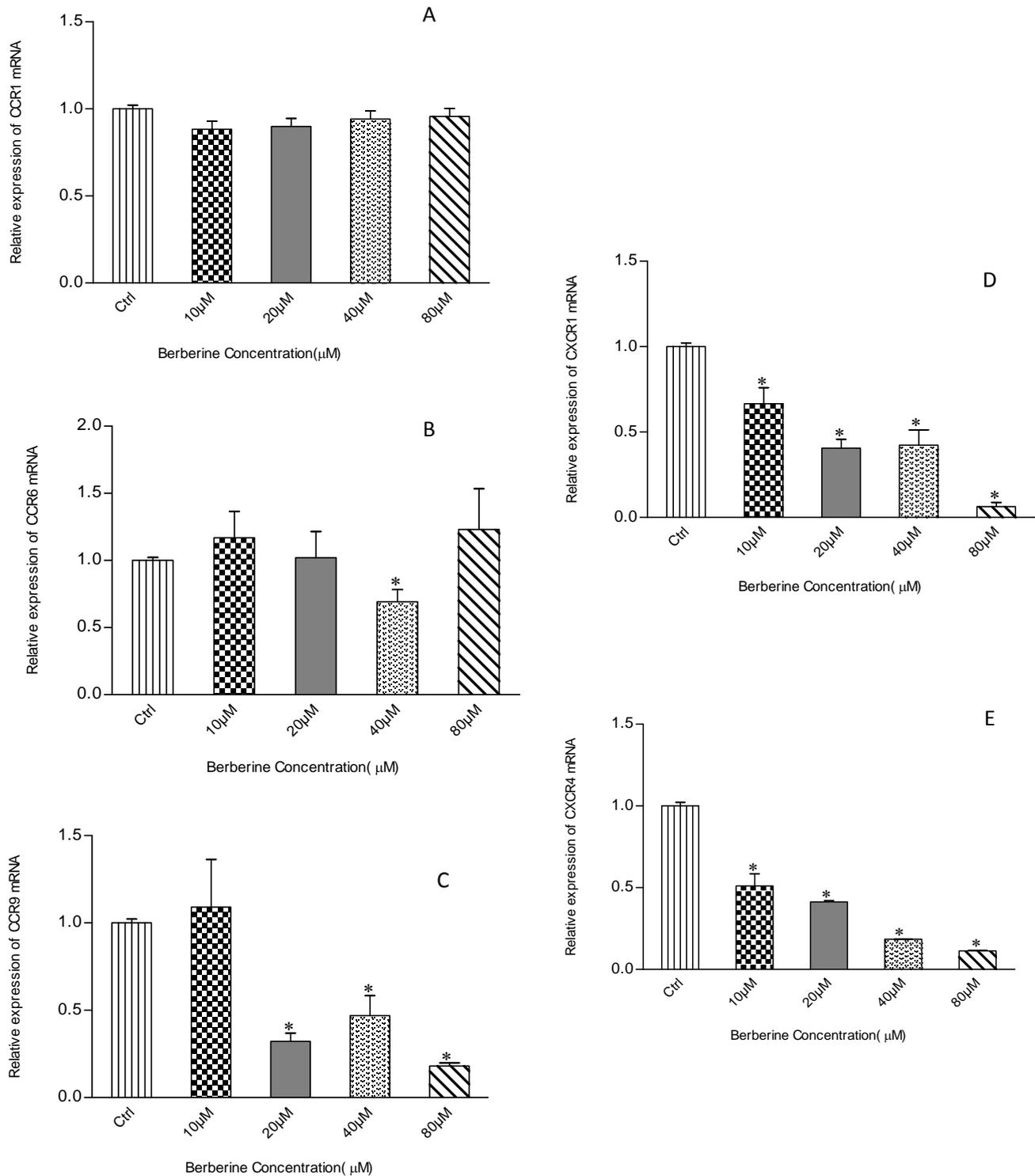


Figure 3. Effects of berberine on the gene expression of chemokine receptors. MCF7 cells were treated with berberine at concentrations of 0, 10, 20, 40 and 80 μM for 24 hr, then, subjected to real-time PCR analysis for determination of alterations in gene expression of selected chemokine receptors including CCR1 (A), CCR6 (B), CCR9 (C), CXCR1 (D), and CXCR4 (E). Data were presented as mean \pm SD from three independent experiments. Results were statistically evaluated by using one-way ANOVA with *Post hoc* test. * $P < 0.05$ was considered significant as compared to control

Furthermore, it was demonstrated that targeted down-regulation of CXCR4 expression in breast cancer cell line via different strategies like RNA interference (RNAi) or CXCR4 antagonists, significantly decreased metastasis of breast cancer cells (21). It has also been reported that CXCL12-CXCR4 axis stimulates the natural selection of breast cancer cell metastasis (22). CXCR4 receptors ligands stimulate small GTPases and induce reorganization of the actin cytoskeleton, which indicates that these receptors might be involved in the migration and, perhaps, metastasis of tumor cells (23). In the present study, decreased migration of breast cancer cell line following berberine treatment was observed with decreased expression of CXCR4. These observations may offer an understanding of the molecular mechanisms that lead to decreased breast cancer migration following berberine treatment.

The activation of NF- κ B occurs as a consequence of activation of chemokine receptors. It has also been shown that tumor angiogenesis, growth and metastasis are facilitated by the transcription factor of NF- κ B which modulates chemokine genes (23). Treatment of melanoma cells with caffeic acid phenethyl ester, an inhibitor of NF- κ B, resulted in an inhibitory effect on cell migration (24). Furthermore, previous experiments on MCF-7 breast cancer cells showed that berberine triggers apoptotic processes through formation of P53 and ROS, over-expression of p21 and inhibition of NF- κ B and Bcl-2 expression (25). These observations support the hypothesis that down-regulation of NF- κ B target genes as a consequence of decreased chemokine receptors expression, may contribute to berberine-induced inhibition of cell migration.

Moreover, it is interesting to know that berberine can inhibit Ras/MAPK and PI3K pathways, which play a major role in promoting gene expression and might be excessively activated in cancer cells (13). PI3K signaling can also be activated by the G-protein-coupled chemokine receptor CXCR4, which seems to be the principal chemokine receptor expressed on cancer cells (26). Therefore, chemokine-induced activation of PI3K may allow for enhanced survival and metastasis of cancer cells. Moreover, it was revealed that dysregulation of the CXCR4-CXCL12 axis in melanoma, activates cell cycle progression and migration via stimulation of MAPK pathway (27). A better understanding of the gene networks and signaling pathways regulated by berberine will definitely enable us to have a better understanding of breast cancer pathogenesis and therapy.

Most cancer-related deaths are not the result of primary tumor growth but are rather caused by the spread of cancer to other organs; hence, therapies designed to minimize metastasis are greatly needed. Targeting chemokine receptors is

one of procedures used to reach this goal. For this purpose, neutralizing antibodies, small molecule antagonists, peptide-derived inhibitors and siRNA nanoparticles have been used (28). Despite extensive pre-clinical literature, there has been limited knowledge on cancer clinical trials because safety and efficacy of each of these strategies in the treatment of patients is currently unknown.

Identification of a natural molecule that can specifically target cancer cells with minimal or no toxicity to normal cells would be of great benefit and berberine can be one of promising natural compounds to inhibit the proliferation of cancer cells and to alter key markers involved in development and progress of cancer, directly or indirectly.

Conclusion

The significant finding of the current study is that treatment of MCF-7 breast cancer cells with berberine decreased cell migration and invasion. Moreover, down-regulation of key chemokine receptors in MCF-7 breast cancer cells may probably represent one of the mechanisms of anti-metastatic effect of berberine. These data may be helpful for future use of berberine in clinical trials for cancer chemotherapy.

Acknowledgment

Thanks to Mojgan Fazli and Piraste Norouzifor for providing technical support. This work was supported in part by a grant (grant no. 9247) from Shahroud University and also a grant, (grant no. 710) from ACECR, Mashhad Branch.

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