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Original Research Article

Involvement of TNF-alpha and IL-10 in breast cancer and patient survival

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Abstract

Purpose: *To investigate the involvement of tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10) in the pathogenesis of breast cancer in vivo as well as the activity of ten Chinese herbal compounds in human breast cancer (MCF-7) cell proliferation in vitro.*
Methods: *In the in vivo study, the association of serum TNF-α and IL-10 with breast cancer cell invasiveness and prognosis was determined in female patients (n = 192) with breast cancer, while in the in vitro study, ten herbal Chinese compounds were screened for their effectiveness against MCF-7 cells. The levels of TNF-α, IL-10, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2/neu) were assayed using their respective enzyme-linked immunosorbent assay (ELISA) kits. Molecular docking was used to determine the lead compound(s) that can effectively inhibit TNF-α and IL-10.*
Results: *Raised serum levels of TNF-α and IL-10 were significantly associated with breast cancer cell invasiveness and poor prognosis ($p < 0.05$). Moreover, there was a strong association between breast cancer prognosis and the expression levels of ER, PR and HER2/neu. Serum TNF-α and IL-10 levels were significantly elevated in stages II and III patients and in those with lymph node metastasis. Treatment of MCF-7 cells with the herbal compounds significantly reduced the synthesis and release of TNF-α and IL-10 ($p < 0.05$). The results of molecular docking showed that baicalein and oridonin significantly inhibited TNF- α and IL-10. The two herbal compounds had the highest docking scores for inhibition of cytokines, as well as favorable interaction energies.*
Conclusion: *These results indicate that TNF-α and IL-10 are involved in the pathogenesis of breast cancer, and that baicalein and oridonin effectively inhibit the proliferation of the cells.*

Keywords: *Baicalein, Breast cancer, Interleukin 10, Oridonin, Tumor necrosis factor alpha*

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INTRODUCTION

Breast cancer, the most frequently diagnosed life-threatening cancer in women, is the leading cause of cancer death among women [1]. According to World Health Organization (WHO) reports, in 2005 alone, about 1.2 million people were diagnosed with breast cancer [2]. Breast cancer is about 100 times more common in women than in men, although males tend to have poorer outcomes due to delays in diagnosis [3]. Over the past two decades, work on breast cancer has broadened our understanding of the disease, resulting in more efficient and less toxic

treatments. Most breast cancers begin in the cells that line the ducts (ductal cancers). Some begin in the cells that line the lobules (lobular cancers), while a few start in the other tissues [1]. The pathogenesis of breast cancer is not well understood, but studies suggest that it may be due to hormonal changes, genetic factors, and environmental or physiological factors [4]. It has also been postulated that breast cancer may be due to complex interactions between environmental and genetic factors. Moreover, obesity and weight gain also influence the occurrence of the disease [5,6]. Like other cancer cells, breast cancer cells require high level of glucose for their metabolism. Even though the prognosis of breast cancer has drastically improved since the last decade, patient survival remains very low [7]. Therefore, improved or targeted therapies are urgently needed to combat metastatic breast cancer [8].
Tumor necrosis factor α (TNF-α) and IL-10, two key molecules in inflammatory responses, cell organization and innate immunity, participate in the pathogenesis of breast cancer [9]. As a cytokine involved in systemic inflammation, TNF-α plays a key role in acute phase reaction. It is produced mainly by activated macrophages and monocytes, and it is required for cell growth, differentiation and survival [10]. Tumor necrosis factor α (TNF-α) is thought to play a vital role in cancer cell proliferation [11]. Overexpression of TNF-α in breast cancer cells has been shown to be associated with lymph node metastasis and tumor invasion [12].

Interleukin 10 (IL-10) is a cytokine with multiple, pleiotropic effects on immuno-regulation and inflammation. It downregulates the expression of T helper 1 (Th1) cytokines, major histocompatibility (MHC) class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. Interleukin 10 (IL-10) blocks NF-κB activity, and it is involved in the regulation of JAK-STAT signaling pathway [13]. The gene that encodes IL-10 is located on chromosome 1 (1q31–1q32), and it is produced by regulatory T and helper T cells [14]. It has been reported that upregulation of IL–10 expression reduces the chances of survival of breast cancer patients [15, 16]. Moreover, IL-10, an inhibitor of nuclear factor kappa-B kinase (IKK) and TNF-α, is known to be associated with the initiation and progression of breast cancer [17]. The aim of this study was to investigate the involvement of TNF-α and IL-10 in the pathogenesis of breast cancer *in vivo* as well as the effectiveness of ten Chinese herbal compounds on MCF-7 cell proliferation *in vitro*.

EXPERIMENTAL

Materials

Tumor necrosis factor α (TNF-α) and IL-10 ELISA kits were products of R&D Systems Inc. (China). Methotrexate was purchased from Sigma-Aldrich (China). Human breast cancer cell line (MCF-7) was obtained from American-Type Culture Collection (USA), while RPMI-1640 and Dulbecco's modified Eagle medium (DMEM) were bought from Invitrogen (USA).

General patient profile

Female patients (n = 192) with breast cancer were recruited over a 3-year period for the *in vivo* study. Breast cancer was confirmed via pathological or cytological diagnosis. Clinical staging was performed according to the Federation of International Gynecology and Obstetrics (FIGO) cancer staging system. The control group comprised healthy patients (n = 50) with no history of breast cancer or any other type of tumor. The study protocol was approved by the Clinical Research Ethics Committee of The Second Hospital of Lanzhou University, Lanzhou City, Gansu Province, China (Nos. ERC/GS/6543-12A and GPPH032016T-21C). The study procedures were carried out in accordance with Helsinki Declaration [18]. Written informed consent was obtained from all the patients before the commencement of study.

Hormone receptor status analysis

Hormone receptor status analysis was carried out to determine whether the breast cancer was responsive to ER, PR or HER2/neu.

Determination of serum TNF-α and IL-10 levels

Tumor necrosis factor α (TNF-α) and IL-10 levels were determined in patients' serum using ELISA.

Cell line and culture

Human breast cancer (MCF-7) cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air until the cells attained 80 % confluency. The medium was replaced with fresh one every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), cultured again, and passaged for later use. The cells were treated with serum-free medium and Chinese herbal

compounds for 24 h. The herbal compounds were astragaloside IV, berberine, camptothecin, magnolol, toosendanin, ginsenoside, oridonin, tanshinone IIA, baicalein and celastrol. Methotrexate served as standard drug. Normal cell culture without crude drugs served as control group. Cells in logarithmic growth phase were selected and used in this study.

Cell viability assay

The viability (proliferative capacity) of MCF-7 cells in the presence of Chinese herbal compounds was determined using 3-(4, 5-dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. The MCF-7 cells were seeded in 96-well plates at a density of 2×10^8 cells/well and cultured in DMEM for 24 h. Then, 20 µM of each of the herbal compounds was added separately to the cells and incubated for 3 days. At the end of the third day, 20 µl of 5 g/L MTT solution was added to the wells, followed by incubation for another 4 h. Thereafter, the medium was replaced with 150 mL of 0.1 % dimethyl sulfoxide (DMSO) solution and agitated at 50 oscillations/min for 10 min. Finally, the absorbance of the samples was read in a microplate reader at 540 nm.

Determination of effect of herbal compounds on TNF-α and IL-10 levels in MCF-7 cells

Cell suspension resulting from the trypsinization of adherent confluent cells was centrifuged at 7000 rpm for 25 min to obtain clear supernatant. The levels of TNF-α and IL-10 in the cell supernatant were assayed using their respective commercial ELISA kits.

Molecular docking of herbal compounds against TNF-α and IL-10

Molecular docking of the herbal compounds was performed against TNF-α (PDB Code: 5MU8) and IL-10 (PDB Code: 2H24) using MVD 6.0. The active sites of both proteins were detected using inbuilt cavity detection program and the binding constraints were set at these sites. The docking engine was set for flexible docking with a strength of 0.90 and a tolerance of 1.10 for residues near the vicinity of the active sites. A total of 200 runs were performed and the best pose was taken for further analysis.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (20.0). Groups were compared using Student's *t*-test. Correlation analysis was carried out using Pearson's correlation coefficient. Statistical significance was assumed at $p < 0.05$.

RESULTS

Serum levels of TNF-α and IL-10 in patients

The level of TNF-α was significantly higher in sera of stage III breast cancer patients than in those of stages I and II ($p < 0.05$). Serum TNF-α level was also significantly higher in patients with metastatic breast cancer than in those without metastasis ($p < 0.05$). However, there were no significant differences in TNF-α levels among patients expressing ER, PR and HER2 ($p > 0.05$).

The levels of 1L-10 were significantly higher in sera of patients in stages II and III than in sera of stage I patients, and they correlated with lymph node metastasis ($p < 0.05$). Moreover, IL-10 level was significantly higher in HER2-positive patients than in PR-positive and ER-positive patients ($p < 0.05$). These results are shown in Table 1. There were only a few viable cells in cultures treated with oridonin, tanshinone IIA, baicalein, astragaloside IV, camptothecin and ginsenoside,

Table 1: Demographic characteristics of patients

Group/Type	Population (n = 192)	Percent (%)	Serum TNF-α (pg/mL)	Serum IL-10 (pg/mL)
Control	50	-	21	46
HER2 positive	83	43.23	31	118
PR positive	109	56.77	28	83
ER positive	94	48.96	34	72
Clinical stage				
I	43	22.40	24	49
II	91	47.40	29	116
III	58	30.21	61	147
Metastasis				
Present	129	67.19	52	114
Absent	63	32.81	23	63

Effect of herbal compounds on viability of MCF-7 cells

relative to methotrexate group, but cell viability was significantly increased in cultures treated with celastrol, toosendanin, magnolol and berberine ($p < 0.05$; Figure 1).

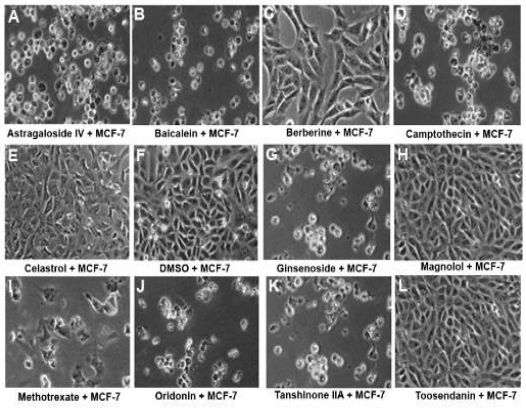

Figure 1: Effect of herbal compounds on the morphology of MCF-7 cells

Levels of TNF-α and IL-10 in MCF-7 cell suspension treated with herbal compounds

Treatment of MCF-7 cells with the herbal compounds significantly reduced the synthesis and release of TNF-α and IL-10 ($p < 0.05$; Table 2). The inhibition of TNF-α release followed the order: baicalein > oridonin > astragaloside IV > tanshinone IIA > camptothecin > berberine > celastrol > ginsenoside > toosendanin > magnolol. Similarly, the inhibition of IL-10 release was in the order: oridonin > baicalein > tanshinone IIA > astragaloside IV > ginsenoside > camptothecin > celastrol > berberine > magnolol > toosendanin.

Table 2: Levels of TNF-α and IL-10 in MCF-7 cell suspension treated with herbal compounds

Group	TNF-α (μM)	IL-10 (μM)
DMSO	300.00 ± 0.14	285.00 ± 0.17
Methotrexate	9.18 ± 0.00	11.73 ± 0.00
Positive control	15.11 ± 0.00	17.52 ± 0.16
Negative control	48.34 ± 0.00	54.79 ± 0.01
Astragaloside IV	9.12 ± 0.00	15.86 ± 0.00
Berberine	21.45 ± 0.00	25.67 ± 0.00
Camptothecin	13.67 ± 0.00	17.24 ± 0.00
Magnolol	64.79 ± 0.04	57.23 ± 0.00
Toosendanin	56.65 ± 0.04	78.78 ± 0.05
Ginsenoside	37.54 ± 0.03	16.63 ± 0.00
Oridonin	8.45 ± 0.01	9.78 ± 0.04
Tanshinone IIA	11.78 ± 0.00	13.75 ± 0.00
Baicalein	6.17 ± 0.02	11.38 ± 0.00
Celastrol	28.34 ± 0.01	23.62 ± 0.03

Docking scores of herbal compounds

The results of molecular docking (Table 3, Figure 2 and Figure 3) showed that baicalein and

Table 3: Docking scores of herbal compounds against TNF-α and IL-10

Ligand	Mol. docking score	Rerank score	Interaction (kJ/mol)	H-bond	Total kJ/mol
TNF-α					
Baicalein	-97.29	-61.39	-110.34	-7.24	276.26
Oridonin	-93.55	-53.99	-106.56	-11.90	-266.00
Astragaloside IV	-75.03	-35.87	-104.66	-16.67	232.23
Tanshinone IIA	-48.50	4.26	-61.55	-1.67	107.47
Camptothecin	-19.51	-3.91	-45.70	0.00	-69.12
Methotrexate (control)	-66.22	124.35	-87.09	-4.14	-33.10
Berberine	-9.38	91.82	-33.60	0.00	48.84
Celastrol	-23.86	304.87	-47.80	0.00	233.21
Magnolol	34.38	266.17	7.98	1.12	309.65
Toosendanin	28.76	288.91	18.99	-2.49	334.17
Ginsenoside	-33.56	540.68	-49.89	-8.53	448.70
IL-10					
Oridonin	-73.66	-54.33	-86.66	0.00	-214.65
Baicalein	-71.23	-52.46	-85.93	0.00	-209.62
Tanshinone IIA	-65.45	-51.19	-91.85	0.00	-208.49
Methotrexate (control)	-60.45	-36.96	-74.13	-8.01	-179.55
Ginsenoside	-104.23	22.67	-93.14	-4.61	-179.31
Camptothecin	-64.23	-6.01	-90.78	0.00	-161.03
Astragaloside IV	-53.36	-23.64	-37.75	-4.93	-119.68
Berberine	-56.01	24.26	-80.20	0.00	-111.95
Toosendanin	11.18	179.14	-13.25	-3.52	173.56
Magnolol	36.38	290.19	20.44	-2.50	344.51
Celastrol	26.73	432.66	15.10	-1.66	472.83

oridonin significantly inhibited TNF- α and IL-10. The two lead compounds had the highest docking scores for inhibition of the two cytokines and favorable interaction energies.

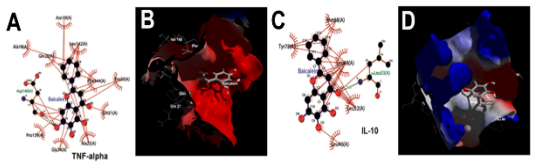

Figure 2: Docking interactions between baicalein and cytokines. **(A):** Amino acid interaction at the active site of TNF-α; **(B):** electrostatic interactions at the active site of TNF-α; **(C):** amino acid interactions at the active site of IL-10; and **(D):** electrostatic interactions at the active site of IL-10

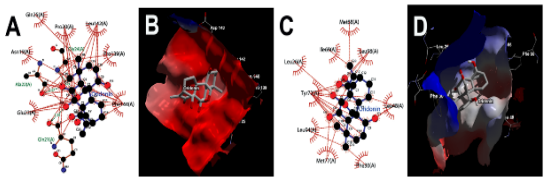

Figure 3: Docking interactions between oridonin and cytokines. **(A):** Amino acid interaction at the active site of TNF-α; **(B):** Electrostatic interaction at the active site of TNF-α; **(C):** Amino acid interaction at the active site of IL-10; and **(D):** Electrostatic interaction at the active site of IL-10

DISCUSSION

Breast cancer refers to cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Globally, breast cancer accounts for 10.4 % of all cancer incidents among women, making it the second most common type of non-skin cancer (after lung cancer) and the fifth most common cause of cancer-related death. In 2004, breast cancer caused 519,000 deaths worldwide (7 % of cancer deaths and approximately 1 % of all deaths) [1,2]. The pathogenesis of breast cancer is not well understood, but studies suggest that it may be due to hormonal changes, genetic factors, and environmental or physiological factors [4]. The 5-year survival for women with invasive breast cancer is 91 %, while the 10-year survival is 84 %. In the event that the cancer is located only in the breast, the 5-year survival is 99 %.

The mixture of cytokines that is produced in a cancer microenvironment has an important role in its pathogenesis. Cytokines are released in response to diverse range of cellular stresses, including carcinogen-induced injury, infection and inflammation. Cancer cells respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis [10,11]. Cytokines function to stimulate a host response that is aimed at controlling cellular stress and minimizing cellular damage. Whereas effective containment of the insult promotes tissue repair, failure to resolve the injury leads to persistent cytokine production and exacerbation of tissue destruction. Thus, host reactions to cellular stress impact on several stages of cancer formation and progression [9]. The present study investigated the involvement of TNF-α and IL-10 in the pathogenesis of breast cancer *in vivo,* as well as the effectiveness of ten Chinese herbal compounds on MCF-7 cell proliferation *in vitro*. The results showed that elevated serum levels of TNF-α and IL-10 were significantly associated with breast cancer cell invasiveness and poor prognosis. Moreover, there was a strong association between breast cancer prognosis and the expression levels of ER, PR and HER2/neu. Serum TNF-α and IL-10 levels were significantly elevated in stages II and III patients as well as in those with lymph node metastasis. Overexpressions of TNF-α and IL-10 in breast tumors have been shown to correlate with lymph node metastasis [19, 20]. In addition, the role of TNF-α in promoting tumor cell metastasis has been reported [21]. Moreover, there is a strong association between high expression level of TNF-α in serum of breast cancer patients and poor prognosis [22]. The results of this study indicate that TNF-α and IL-10 may contribute to breast cancer progression. These findings are in agreement with reports of previous studies [23-25]. Molecular docking results showed that baicalein and oridonin significantly inhibited TNF-α and IL-10. It is likely that TNF-α and IL-10 participate in the pathogenesis of breast cancer. Baicalein and oridonin may inhibit TNF-α and IL-10. Interleukin 10 (IL-10) may function as an inflammatory modulatory cytokine with both anti-tumor and pro-tumor effects.

CONCLUSION

The results of this study indicate that TNF-α and IL-10 participate in the pathogenesis of breast cancer, and that baicalein and oridonin effectively inhibit the proliferation of the breast cancer cells.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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