Abstract: Objective: Given the role of basic fibroblastic growth factor (bFGF) in tumor growth, it has been considered as a potential target for tumor therapy. In this study, we investigate the effect of bFGF antagonistic peptide on the growth and angiogenesis of 4T1 mammary carcinoma tumor (MCT) in BALB/c mice. Methods: An engineered peptide was injected into BALB/c mice in doses of 1, 2.5, 5 and 10 mg/kg daily for 14 days. Immunohistochemical analysis using anti-CD31 and anti-CD34 were conducted as indices of angiogenesis. In addition, blood samples were taken from the eyes of treated and control mice and the levels of Interleukin-8 (IL-8) and Tumor Necrosis Factor-α (TNF-α) were measured by ELISA. Data was analyzed by ANOVA using SPSS. Results: The antagonistic peptide inhibited growth and angiogenesis of MCT (P ≤0.05), and decreased the serum level of IL-8 and TNF-α in treated groups compared to the control groups. Conclusion: The inhibition of tumor angiogenesis has been considered as an important strategy to halt tumor growth. The results of current study confirm that the antiangiogenic peptide effectively inhibited the growth of MCT, and shows potential for clinical trials for the treatment of cancer in humans.

Keywords: bFGF, Peptide, Angiogenesis, Breast Cancer, Tumor growth

1 Introduction

Cancer is a major global health issue and the leading cause of death in children and adults [1]. Breast cancer affects millions of women worldwide and is the most common type of malignancy. In western countries, it comprises approximately one-third of all cancers among women. According to statistics in Iran, the risk of breast cancer is one per 10-15 women with the age of onset of at least one decade earlier compared to women in other countries [2]. This is the second cause of death among women [3].

Cancer is a multistage process that includes irreversible genetic changes in stem cells, followed by their clonal proliferation, and finally development of an invasive phenotype and cancer metastasis. Predication and treatment of cancer should be carried out in different stages of this process, using different phenotypes [4]. The etiology of breast cancer is generally considered as multiple genetic predisposition combined with environmental factors. Importantly, the fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) signaling pathway, appears to play a crucial role in the development and progression of breast cancer [5]. As necessary components in this pathway, FGFRs are frequently overexpressed in breast cancer. FGF signaling has been shown to be susceptible to subversion by cancer cells though modulating key pathways such as cell proliferation, differentiation, and survival [6, 7]. Today’s conventional cancer treatments include surgery, chemotherapy, and radiotherapy [8]. In the majority
of cancers, the main cause of failure in treatment is metastasis [9]. Although surgery and radiotherapy are suitable for local tumors, they are not adequately efficient in dealing with metastases, which are often treated using chemotherapy. However, due to its side effects at high doses, chemotherapy has limited applicability [10, 11]. Since chemotherapy drugs have numerous side effects, there has been considerable interest in the development of peptides as a therapeutic for the next generation of anticancer drugs [12, 13].

Cytokines are regulatory proteins or lipoproteins with low molecular weight, and play an effective role in regulating immunological and inflammatory functions of the body [14]. Various types of cytokines are involved in the onset or progression of cancer. Such effects include prevention of inactivation, proliferation or differentiation. They can also provide information on the incidence of cancer and even proliferation and metastasis of cancer cells. Furthermore, they can prevent further cancer progression through their anti-inflammatory and antitumoral effects [15]. Studies have shown that secreted IL-8 by tumor cells, has several pre-inflammatory effects. Excessive expression of IL-8 is followed by increased tumor growth and breast cancer recurrence. Moreover, there is a direct correlation between IL-8 levels and tumor angiogenesis, growth, and metastasis [14]. Chronic inflammation has been shown to play an effective role in carcinogenesis [16]. In the majority of studies, TNF-α has been introduced as a bridge between cancer and inflammation [17, 18].

In the present study, a bFGF antagonistic peptide was administered to BALB/c mice having 4T1 mammary carcinoma tumor (MCT), and we observed that following treatment, the growth and angiogenesis level of tumors was inhibited. Furthermore, serum levels of interleukin-8 was decreased in treated mice compared to the controls.

2 Materials and Methods

2.1 Metastatic breast cancer modeling

Previous studies involving the injection of human tumor cells in mice have been shown to poorly replicate metastasis [17]. The 4T1 cell line ranks as one of the best breast cancer cell line with the capability to spontaneously metastasis to multiple areas within the body, replicating the affected sites in human breast cancer [19, 20].

The 4T1 breast cancer cell line with BALB/c origin was purchased from the Pasteur Institute of Iran. These cells were cultured in high glucose DMEM culture medium containing 10% fetal bovine serum (FBS), 5% non-essential amino acids and penicillin and streptomycin. Cells were incubated at 37°C containing 5% CO₂. The BALB/c mice were maintained for 5-7 weeks at a temperature of 24°C with 12 h light/dark cycle with free access to food and water.

To induce breast cancer, the 4T1 cell was firstly injected into mice and left to grow. The cancer tissue was then transplanted to other mice. The protocol was as follows:

At day 0, the injection point was disinfected with cotton wool and alcohol. Cancer cells was injected subcutaneously at 10⁵×7 cells for all mice. The injection point was adjacent to the lowest right side of the breast gland. After the tumor reached adequate size, it was then transplanted into other mice.

For cancer cell transplant: After euthanasia of mice, the tumor pieces with approximate dimensions of 3×3×3 mm were removed and were implanted subcutaneously to the back right area of mice. The implant area was closed using a special clip on the skin. Approximately 2 weeks after transplantation, the tumor reached a size of 0.5 to 1 mm³. At this time, treatment started with the peptide. This research was carried out in accordance with the ethical guidelines of research on the experimental animals of the National Ethics Committee in Biomedical Research of the Islamic Republic of Iran.

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

2.2 Peptide designation

Based on the functional amino acids of basic fibroblast growth factor (bFGF), a peptide was designed with the sequence of CGGSGLPLGHIKC. The designed peptide is expected to bind to bFGF receptor-1. The peptide was synthesized by Shine Gene Bio-Technologies, Inc. (Shanghai, China). The antiangiogenic and antitumor properties of the peptide and its effect on the level of interleukin-8 was evaluated in the BALB/c mice bearing 4T1 MCT. For this purpose, mice were randomly divided into 6 groups with 10 members per group. Four treatment groups received the peptide (bFGF) in 4 different doses (1, 2.5, 5 and 10mg/kg) daily for 14 days intraperitoneally; the negative control group was treated with PBS and the positive control group was injected with Doxorubicin.
2.3 Viability analysis using MTT method

MTT is a colorimetric method used to determine the viability and cell toxicity of materials. It is based on the reduction of yellow 4, 5 Dimethyl thiazole, 25 Diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase enzyme in living cells. This reaction results in the formation of insoluble formazan crystals. These crystals are solubilized and measured at an optical absorption at 570nm using an Ultraviolet-Visible Spectrophotometer. Proliferation of SKOV3 ovarian cancer cells is dependent on bFGF. The antagonistic property of the peptide was investigated on SKOV3 cells in the presence of differing concentrations of the peptide (50 to 600 ng/ml) and 20 ng/ml of rhbFGF. SKOV3 cells were seeded in 96-well plates with 5 × 10³ cells per well and were incubated at 37°C and 5% CO₂. Cells were washed with PBS and serum-starved in serum-free DMEM with 0.02% FBS for 24h. Cells were then treated with serially diluted peptides, 20 ng/ml bFGF for 24h. The insoluble formazan crystals were solubilized through addition of Dimethyl sulfoxide (DMSO). The absorption wavelength was read at 570 nm and number of viable cells was determined (FIG2). The MTT assay is currently the most extensively used method for half –maximal (50%) inhibitory concentration (IC50) measurements.

2.4 Peptide Administration

The bFGF antagonist peptide was injected daily in doses of 1, 2.5, 5 and 10 mg/kg for 14 days in BALB/c mice bearing the 4T1-MCT. The injection was started when the tumor size reached approximately 400 mm³.

2.5 Tumor size measurement

In order to analyze the changes in the growth of the tumor, the tumor size was measured on alternate days until the end of the treatment period. The length and width of tumor was measured each time before injection and the tumor size was measured using (length × width² × 0.5) formula.

2.6 Immunohistochemical studies

The mice were examined every day in terms of physical and behavioral traits and were sacrificed after 14 days treatment (day 28 after implantation). After removing the tumor from their body, half of the tumors were fixed with 10% formalin (Sigma Aldrich, product number HT501128). Following fixation, all tumors were impregnated with paraffin and were cut using a microtome and placed on a slide with a thickness of 2 micron. To analyze the angiogenesis process, anti-CD34 antibody (1:50, clone QBEND-10 M7165 DAKO Glostrup Denmark) and anti-CD31 antibody (M 0823 DAKO Glostrup Denmark) were used as indices of tumor angiogenesis. Immunohistochemistry was done using the streptavidin biotin peroxidase complex method according to manufacturer’s instructions. Firstly, paraffinized tumors were deparaffinized. To remove the peroxide activity, Blocking Reagent andogen (3% Oxygenated water in methanol) was used. For antigen retrieval, samples were heated with a microwave over for 15min. Samples were stained with the desired antibody and DAB was used as a chromogen.

2.7 Measurement of IL-8 and TNF

Before mice were sacrificed, blood samples were collected from their eyes and the serum was separated. The production of IL-8 and TNF was measured using either the mouse Interleukin 8 (IL-8) or mouse TNF–α ELISA Kit.

2.8 Statistical analysis

The results are expressed as the mean values ± S.E. Comparison of multiple group means in in vitro functional studies and in vivo tumor growth studies was performed using SPSS and one-way ANOVA statistical test. Where the differences were significant, post-hoc (Tukey) was applied to determine where the difference was occurring. The immunohistochemical data were analyzed by the Student t-test. P < 0.05 was considered statistically significant.

3 Results

3.1 Inhibition of tumor growth

To evaluate whether bFGF had any antitumor effect, the size of the induced MCT growth was measured. The peptide was administered to BALB/c mice by i.p in three doses: 1, 2.5, 5 and 10 mg/kg/day. The results obtained from peptide-treated samples were compared with each other and with the PBS-treated control group. Tumor size data was analyzed using SPSS, and one way ANOVA test with p<0.05 considered as significant (Figure 1).
3.3 Measurement of Serum IL-8 Level

Results showed that peptide therapy reduces the blood concentration of IL-8, which is an angiogenesis activator. The serum IL-8 level was 33.20±3.74 pg/mL in the PBS-treated control group and 12.45±2.51 pg/mL in the peptide-treated group, and this was statistically significantly.

3.2 Cell proliferation

The results showed that treatment with peptide for 24 h resulted in a dose-dependent decrease of SKOV3 cell proliferation. Accordingly, the peptide interferes with the stimulatory effect of rhbFGF and act as its antagonist.
3.4 Measurement of Serum TNF-α Level

Results showed that the serum level of another angiogenesis-related factor TNF-α was 0.0047±0.0024 pg/mL in peptide-treated group and 0.0426±0.0074 pg/mL in the control group. There was no significant difference observed between the groups before the intervention.

3.5 Immunohistochemical studies for CD31, CD34

To further assess the mechanism of peptide action, histopathological analysis of angiogenic indices CD31 and CD34 performed on the peptide- and PBS-treated tumor sections after 14 days of peptide administration.

The presence of proteins associated with each staining was visualized by the brown color on slides. The staining intensity level was reported as either mild, intermediate or strong. Chi-squared statistical analysis (P<0.05) of samples and control group was done (Figure 3).

4 Discussion

Current cancer treatment strategies is followed with many complications. This includes damage to healthy cells and drug resistance, which results in its recurrence. As a result, it is essential to develop new therapeutic approaches with fewer side effects, such as peptide therapy. Folkman was among the leading researchers who recommended the inhibition of tumor blood vessel formation as a form of

![Figure 3: Effects of peptide on tumor angiogenesis in 4T1 tumor allograft. Tumors were subjected to Immunohistochemical staining for CD31 (A) CD34 (B), and H&E (C) staining. A representative picture has been shown for each treatment group in each case. CD31, CD34 positive cells were calculated by the number of positive (brown) cells ×100/total number of cells count in 10 randomly selected areas in each tumor sample (P≤0.05).]
cancer treatment [21]. Regarding the high selectivity and low cost of peptides, these compounds serve as a highly suitable candidate, fulfilling the requirement of a new therapeutic with fewer side effects and lower toxicity [22].

Due to increased cancer resistance to conventional treatments, Khumalo et al. (2005) recommended that new cancer factors should be discovered and identified to increase the sensitivity to cancer cells [23]. Choren et al. (2007) provided a report about the first FDA-approved anti-angiogenic drugs for the treatment of solid tumors and inhibition of vascular endothelial growth factor [24]. The synergistic effects of combined chemotherapy were reported by Stup et al. in 2010 [25]. Also, Anderson et al. (2010) reported this synergistic therapy in combination with radiotherapy [26]. It was reported by Li et al. (2012) a type of peptide that inhibited bFGF proliferation in breast cancer cells and likely induced cell-cycle arrest in the G0/G1 phase [27]. In 2015, Macrovich et al. recommended the use of new drug delivery techniques, development of synergistic methods, and application of preclinical animal models for anti-angiogenic therapies [28].

This study investigated the effect of an engineered peptide on angiogenesis and growth of a highly invasive and metastatic breast cancer; 4T1-MCT. Our results indicate that peptide treatment was followed by regression of tumor growth. The difference in this reduction was significant between the negative control group with peptide group and positive control (doxorubicin).

Many studies into patients with breast cancer showed a reduction in immune response and cytolytic function. This results in reduced proliferation in immune cells and an increased incidence of cancer [29]. Interleukin-8 (IL-8), a potent chemoattractant, has been demonstrated to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor [14]. Studies have shown that IL-8 is secreted by tumor cells and has several pre-inflammatory effects. Elevated expression of IL-8 is followed by increased tumor growth and breast cancer recurrence. Moreover, there is a direct correlation between IL-8 levels and tumor angiogenesis, growth, and metastasis [14]. We observed that bFGF peptide therapy led to a significant reduction of serum IL-8 levels, which confirms the inhibitory effect of peptide on the tumor growth.

Another important pre-inflammatory cytokine is TNF-α. It plays a critical role in the growth, differentiation, performance, and survival of many cells, and is produced by many cells such as macrophages, neutrophils, fibroblasts, lymphocytes, and tumor cells [30]. High and abnormal levels of TNF-α have been reported in the serum of patients with cancer [31]. These studies have shown that higher TNF-α levels are correlated with tumor deterioration. It is likely that TNF-α plays a role in the pathogenesis and progression of cancer. In the majority of studies, TNF-α has been introduced as a bridge between inflammation and cancer [14, 15]. This factor not only results in tumor growth, but also leads to necrosis and apoptosis. Mantovani et al. (2000) reported that the administration of high-doses of TNF-α, functions as a tumor suppressor. In contrast, when secreted by tumor cells, it functions as a tumor promoter and results in proliferation, migration, angiogenesis, aggression, and metastasis of cancer cells [29]. Our results showed that following treatment of mice with peptide, this caused a decrease in serum TNF-α levels.

5 Conclusion

Our results indicated that bFGF antagonistic peptide can inhibit the growth of 4T1-MCT through inhibition of tumor angiogenesis and causes a decrease in IL-8 and TNF-α serum levels. These observations reveal that anti-growth factor agents have a great potential for the treatment of cancers. In addition, we showed that peptides are amenable alternatives to antibodies and other chemical compounds have limitations in cancer therapy.

Conflict of interest: Authors state no conflict of interest.

References

[8] Trosko JE, Chang CC. Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and


