Evaluating Serum Level of Granulocyte, Macrophage and Granulocyte-Macrophage Colony-Stimulating Factors in Patients with Breast Tumor

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ABSTRACT

Colony-stimulating factors (CSFs) are glycoproteins that stimulate the proliferation and differentiation of hematopoietic progenitor cells in the bone marrow. But numerous studies have shown that these factors can stimulate the proliferation of non-hematopoietic cells, including cancer cells. Hence, in this study, Macrophage-CSF (M-CSF), macrophage-granulocyte CSF (GM-CSF), and granulocyte-CSF (G-CSF) were evaluated in the serum of patients with breast tumors and their relationship with pathological and paraclinical parameters of the disease. In this study, 62 patients with breast cancer who had not received any treatment and 54 healthy women who matched the age group with the patient group were included as a control group. After obtaining informed consent, 5ml of peripheral blood was taken from both groups, and their serum was isolated. Serum levels of the studied cytokines were measured by the cytokine-bead array method. Data were analyzed using SPSS18 software and a significance level of 0.05. The mean serum levels of M-CSF, G-CSF, and GM-CSF growth factors in patients with breast cancer were 63.48, 16.13, and 6.11pg/ml, respectively. Although the statistical analysis did not show a significant difference between serum levels of these growth factors in the patient and control groups (p >0.05), further studies showed that with increasing disease stages from I to III, serum levels of GM-CSF significantly. Decreases (p = 0.016). Overall, the results of this study indicated the antitumor role of GM-CSF in breast cancer. However, confirmation of these results requires more complete studies with larger sample sizes.

Introduction

Tumor cells and cells that are part of the tumor microenvironment (e.g. vascular cells, fibroblasts, and inflammatory cells) are constantly exposed to growth factors and cytokines that affect their growth, invasion, and metastasis (1). On the other hand, tumor cells affect endothelial cells, macrophages, T cells, and fibroblasts by expressing markers and producing multiple factors to escape host defense, perform angiogenesis, and stimulate growth, survival, and metastasis; therefore, they stimulate tumors (2).

Colony-stimulating factors (CSFs) are glycoproteins that stimulate the proliferation and differentiation of hematopoietic progenitor cells in the bone marrow (3). These factors, which are produced by different types of immune and non-immune cells, include macrophage stimulatory factor macrophage factor M-CSF or GM-CSF, CSF1 or G-CSF, CSF2 or CSF3, which are essential factors in survival, proliferation, differentiation, maturation, and activation of hematopoietic cells, including monocytes and macrophages, are known (4). Studies have also shown that these growth factors can stimulate the proliferation of non-hematopoietic cells, including cancer cells (5, 6).

There are numerous reports that many tumor cells, including prostate (7), lung (8), colorectal (9), and endometriosis (10), are also capable of producing these factors. It indicates the critical role of clonal stimuli, especially GM-CSF, M-CSF, and CSF in tumor biology (11). Part of this behavior can be attributed to the stimulation of monocytes, macrophages, and dendritic cells and their effect on the antigen delivery process and immune response (12). Also, by various mechanisms, these factors, including stimulation of angiogenesis, provide

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conditions for tumor growth or cause invasion and metastasis in different categories of cancer (13).

However, the amount of these factors has been measured in different ways and sometimes with low accuracies, such as ELISA. Therefore, in this study, we looked at these factors together with a new and much more accurate cytokine bead array method that can measure much lower values than conventional methods such as ELISA. We also examined their relationship to each other and paraclinical and pathological factors.

**Materials and methods**

**Samples**

In a case-control study, 62 women with breast cancer with a mean age of 48.37 ± 11.78 years whose cancer was confirmed by a physician and pathological examination were included in this study. Also, 54 healthy women who were age-matched to the patient group (45.07 ± 8.93 years) and did not have any cancer and autoimmune diseases in themselves and their first-degree families were also selected as the control group.

After conscious consent, 5ml of peripheral blood was taken from the patient and control groups. After 30 minutes of blood clotting, it was placed in a centrifuge at 1000Xg for 10 minutes. The obtained sera were then stored in 30μl volumes at -70°C until the test.

**Measurement of serum levels of growth factors G-CSF, M-CSF, and GM-CSF**

Serum levels of M-CSF, G-CSF, and GM-CSF cytokines were measured by cytokine bead array method based on the protocol of Biolegend, USA (Biolegend), a small single-bead immunoassay method. This method works using the same basic principles of the safety sandwich (bead- cytokine-antibody) and thus traps a soluble analyte between two antibodies. The population of beads is of different sizes, and they have different amounts of fluorescent light that allow them to be clearly distinguished from each other. In addition, each set of beads is conjugated to its surface with a specific antibody, which makes it act as a recipient of a particular analyte. Finally, for each population of moths, the intensity of the fluorescent signal is measured using a flow cytometer (Figure 1).

**Figure 1. Flow cytometry reading chart of clonal stimuli by cytokine-bead-array method (CBA)**

In this method, the serum was briefly diluted with a reaction buffer ratio of one to two and then poured into the corresponding tubes. Then 25μl of reaction buffer and 25μl of microbead were added to each tube. Then 25μl of the detection antibody (Ab) was added to the respective tubes. In standard tubes, at the same time as the test tubes, first 25 microliters of matrix B and then 25 microliters of ETA 7 standards were added to each tube. Standards 1 to 7 were
prepared based on the kit instructions from the standard solution in the kit. 25 μl of Matrix B was added to the standard tubes, and the steps of adding a bead and determining antibodies were performed in the standard tubes. Then the test and standard tubes were placed on a shaker at 500 rpm for two hours. Then 25 μl of SAPE solution was added to each tube, and the tubes were placed on the shaker again for another 30 minutes. The tubes were then washed twice with 200 μl of the wash solution, and finally, 200 μl of the wash solution was added to each tube and read with a four-color flow cytometer BD Biosciences, USA (FACS Calibur).

Statistical analysis
Serum cytokines were measured according to the relevant kit protocol, and the concentration of each analyte was determined based on a known standard curve using Biolegend, USA (LEGENDplex) software. The type of analysis used in this design was Log Scale (Five) parameter curve fitting. The results of the measurements were analyzed with SPSS 16 software. Since the distribution of samples was not normal, the Mann-Whiney U test was used to compare the relationship between the two groups. Also, the Kruskal-Wallis H test was used to examine the relationship between more than two groups, and Spearman Ranks’ Correlation test was used to examine the relationship between Serum levels of cytokines. A P-value less than 0.05 were considered significant.

Results and discussion
Samples
This study measured serum levels of growth factors M-G-CSF, G-CSF, and GM-CSF among 116 samples, including 62 patients with breast cancer at different stages of the disease and 54 healthy individuals as a control group. All patients were female. Basic patient information is given in Table 1. Most patients were in stage 2 disease (24.64, 38.70%), and none of the patients were in stage 4 at the time of surgery. In the majority of patients, the degree of differentiation of tumor tissue was moderate (II) (26%), and the most common type of tumor was invasive ductal carcinoma (87.1%). Lymph node involvement was also seen in 57.4% of patients.

<table>
<thead>
<tr>
<th>Features</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48.37 ± 11.78</td>
</tr>
<tr>
<td>Condition of lymph nodes</td>
<td></td>
</tr>
<tr>
<td>Free</td>
<td>26 (42.6%)</td>
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<tr>
<td>Involved</td>
<td>35 (57.4%)</td>
</tr>
<tr>
<td>Not reported</td>
<td>1 (1.6%)</td>
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<tr>
<td>Tumor stage</td>
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</tr>
<tr>
<td>I</td>
<td>20 (32.3%)</td>
</tr>
<tr>
<td>II</td>
<td>24 (38.7%)</td>
</tr>
<tr>
<td>III</td>
<td>18 (29.0%)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>54 (87.1%)</td>
</tr>
<tr>
<td>MC</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>ILC</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td>DCIS</td>
<td>3 (4.8%)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
</tr>
<tr>
<td>T1 (&lt;2)</td>
<td>30 (48.4%)</td>
</tr>
<tr>
<td>T2 (2-5)</td>
<td>24 (38.7%)</td>
</tr>
<tr>
<td>T3 (&gt;5)</td>
<td>7 (11.3%)</td>
</tr>
<tr>
<td>Not reported</td>
<td>1 (1.6%)</td>
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<tr>
<td>Degree of histology</td>
<td></td>
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<tr>
<td>Well differentiated</td>
<td>16 (25.8%)</td>
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<tr>
<td>Medium differentiated</td>
<td>26 (41.9%)</td>
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<tr>
<td>Poorly differentiated</td>
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<tr>
<td>Progesterone receptor</td>
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<tr>
<td>Her2 expression</td>
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<tr>
<td>Positive</td>
<td>11 (17.7%)</td>
</tr>
<tr>
<td>Equivocal</td>
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</table>

| Serum levels of growth factors M-CSF, G-CSF, and GM-CSF
In patients and the control group, the minimum and maximum mean and median values measured from the serum level of growth factors in patients with breast cancer and the control groups are summarized in Figure 2. Due to the abnormal distribution of M-CSF, G-CSF, and GM-CSF in the patient and control groups, a non-parametric Mann Whitney U test was used to compare the serum levels of the studied factors, although the statistical analysis showed a significant difference between Serum levels of these growth factors were not shown in the patient and control groups (p = 0.60, p = 0.72, p = 0.67, respectively; Figure 2-A).
In the next stage, serum levels of growth factors M-G-CSF, CSF, and GM-CSF in patients with different
clinical and pathological disease features, including disease stage, tumor grade, and lymph node involvement, were evaluated and compared. A comparison of serum levels of growth factors studied between patients with different stages of the disease showed a close relationship between GM-CSF factor and disease stage (p = 0.056). Further analysis showed the difference between stage one and three diseases (p = 0.016). With increasing the stage of the disease from I to III, the serum level of GM-CSF decreased significantly (Figure 2-B).

A comparison of serum levels of growth factors studied between patients with at least one lymph node involved (patients + LN) and patients without lymph node involvement (-LN) also showed serum G-CSF levels in patients with at least one lymph node is positive and have an increasing the number of trends. However, this increase was not statistically significant (p = 0.09). There was also a significant increase in serum G-CSF levels in patients with nephrotic invasion (p = 0.09). No association was found between any factors and vascular involvement or neurological involvement. Serum levels of growth factors were also assessed between patients with different expressions of hormone receptors, including estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor (HER2).

The results showed that among the studied factors, the serum level of GM-CSF in patients with tumor-positive tissue for PR, compared to patients with tumor PR negative, showed a significant increase (p = 0.056). However, examination of serum levels of this factor and other factors in patients showed no association with different expression statuses of other hormone receptors.

The two-way correlation test between serum levels of growth factors and age in both patient and control groups showed that in both groups, a significant relationship was observed between serum levels of GM-CSF and age. It means that the serum level of GM-CSF increases with increasing age of both patients (p <0.0001) and control (p = 0.02). But no correlation was found between the other two factors and age, neither in the patient group nor the control group. The correlation between serum levels of the studied factors and each other was also investigated. The results showed a strong and direct relationship between serum levels of GM-CSF and two other factors, namely G-CSF and -M CSF, which is statistically significant in all cases (p <0.001). There was also a significant positive relationship between G-CSF and M-CSF factors (p <0.001). This relationship was incremental in both patient and control groups.

Figure 2. Comparison of serum levels of clonal stimuli in patients with breast cancer and healthy individuals (A) and patients in different stages of the disease (B); Results are reported as mean ± SEM. * Means the value of p is less than 0.05

This study evaluated the serum levels of three essential growth factors in hematopoiesis, including M-CSF, G-CSF, and GM-CSF, in patients with breast cancer. Although the statistical analysis did not show a significant difference between serum levels of these growth factors in the patient and control groups, comparing the serum levels of growth factors studied between patients with different stages of the disease showed a significant relationship between GM-CSF and stage of the disease. Serum levels of GM-CSF decreased significantly with increasing disease stage. Serum G-CSF levels also increased in patients with at least one positive lymph node (LN + patients) and patients with lymphatic invasion, although this
increase was not statistically significant. GM-CSF is a glycoprotein with a molecular weight of 22 kDa that is produced by various cell types, including T and B lymphocytes, macrophages, endothelial cells, and fibroblasts, and is one of the most significant growth factors for the survival and maintenance of hematopoietic cell line (12). This study observed that serum GM-CSF levels decreased significantly with increasing disease stage. The decrease observed in this study may indicate suppression of cytokine expression due to tumor growth or the antitumor role of this cytokine in breast cancer. In this regard, GM-CSF has been shown to stimulate monocytes, leading to antitumor behavior. GM-CSF improves antigen delivery to macrophages and the immune response (13).

Several laboratory studies have suggested that GM-CSF can affect monocytes and macrophages and stimulate survival and release of inflammatory agents to kill pathogenic organisms and tumors (14, 15). GM-CSF can also inhibit the growth and metastasis of mouse models of breast tumors by inhibiting the production of angiogenic factors (16). A recent study showed that increased GM-CSF expression increases the sensitivity of the MCF-7 breast cancer cell line to anticancer drugs, including FU5, cisplatin, and doxorubicin (17). In contrast, several reports show that many tumor cells also produce this factor, and its presence can stimulate the growth of tumor cells (18, 19). Although there was no difference between the patient and control groups in our study, an increase in serum GM-CSF levels was reported in patients with lung cancer (20) and colorectal (9), and the presence of this cytokine was considered an indicator of disease progression. The observed results can be attributed to the difference in tumor type or sample size. Another factor examined in this study was the macrophage stimulating factor or M-CSF. Macrophages play an essential role in the development of normal breast tissue, and in cancer, the breast is associated with invasion and poor prognosis of the tumor (21). Tumor-dependent macrophages produce factors such as matrix metalloproteinases and vascular endothelial growth factors that facilitate invasion and angiogenesis (22).

In several studies, M-CSF coding transcripts in tumor cell lines have been demonstrated (23). In addition, tumors with a high percentage of M-CSF-expressing cells are more susceptible to macrophages, and this group of tumors is more invasive and has a poor prognosis (24). Many breast tumors also have M-CSF. In these patients, serum M-CSF levels were associated with tumor size, metastasis, and poor cancer outcomes (25). In one study, M-CSF as a tumor, a suitable marker, was introduced in breast cancer (26). In confirmation, in a mouse model, M-CSF deficiency protected against breast cancer metastasis, and its re-expression in breast tissue restored metastatic cell activity (27). A similar study also showed that serum M-CSF levels were significantly increased in colorectal cancers compared with healthy individuals, and this increase was also associated with disease stage and lymph node metastasis (28). However, in the present study, no difference in serum levels of this cytokine was observed between patients with breast cancer and the control group and clinical and pathological factors of the disease. And colleagues the plasma level of M-CSF factor was evaluated in advanced breast cancer patients (29), while in this study, patients were present in all stages and mainly in stage II of the disease.

The third studied factor was the granulocyte colony-stimulating factor (G-CSF). The presence or increased expression of this cytokine in the tumor has been shown in several studies. Primary and metastatic lung cancers are the most common among cancers stimulated by abnormal G- / GM-CSF secretion (30). Increased G-CSF concentration was also observed in colorectal and breast cancer patients and serum levels. This factor in colorectal cancer was associated with tumor stage in these patients (31). In this study, we also observed that serum G-CSF levels in patients with at least one positive lymph node (patients + LN) and patients with Lymphatic invasion have a relatively increasing trend. However, this increase was not statistically significant, in line with Ku et al. (32).

To stimulate cancer development through its ability to stimulate tumor-dependent angiogenesis (33), SF promotes invasion in the human cell line in the laboratory (25, 33). Studies have shown that breast cancer cells can produce these growth factors in vitro (34). In a study conducted in Poland to evaluate the diagnostic value of G-CSF and compare it with classical tumor markers such as CEA and 9 CA25 in colon cancer, the results showed that this marker helps diagnose colorectal cancer. But this factor had no
value in differentiating cancerous tumors from polyps (35).

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Authors’ contribution
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Interest conflict
The authors declare that they have no conflict of interest.

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Availability of data and materials
The data used to support the findings of this study are available from the corresponding author upon request.

Statements and Declarations
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