The role of angiogenesis in the development of neoplasia has been identified and characterized. However, anti-angiogenic therapeutic intervention still requires more evidence to become recognized and successful. The aim of this study was to evaluate levels of selected proangiogenic factors, such as fibrinogen, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in serum of patients with the gynecologic cancer on the first, third and sixth day of antibiotic therapy, routinely administered as a perioperative treatment. In addition, serum concentrations of $\gamma-\gamma$ dimers and $\alpha$-polymers of cross-linked fibrin structure and the degree of bFGF binding with the fibrin network were investigated. Immunohistochemistry staining of the excised tumor tissue was also performed. We observed higher levels of bFGF, VEGF, as well as fibrinogen in patients with gynecologic malignancy, as compared to healthy women. In cancer patients, the concentration of $\alpha$-polymers and $\gamma-\gamma$ dimers of fibrin network increased. Further only $\gamma-\gamma$ dimers fraction of fibrin was found to bind to bFGF. Immunohistochemical analysis indicated the presence of bFGF in an excised tumor tissue. In conclusion, the decrease of proangiogenic bFGF and fibrinogen levels in a clinical trial of gynecologic patients may confirm anti-angiogenic properties of selected antibiotic therapy.

**Keywords**: bFGF, VEGF, Fibrinogen, Gynecological cancer

Proangiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are responsible for neoplastic processes. The bFGF is a member of the FGF family, which possesses broad angiogenic and mitogenic activities. It is also involved in many biological processes, such as arteriogenesis, limb and nervous system development, wound healing and tumor growth.

VEGF and its different isoforms are directly involved in neoplasia development by inducing tumor cells proliferation in autocrine and paracrine way, leading to metastases and cancer growth. Ability of VEGF to increase vessel permeability also contributes to neoplastic processes by allowing tumor cells penetration to the extravascular space, leading to the development of tumors. On the other hand, cancer cells stimulate biosynthesis of VEGF.

Some types of cancer are richly vascularised due to increased angiogenesis, regulated mainly by VEGF. Intensified VEGF expression and its receptor VEGFR2 influence malignancy of tumor and are dependent on many factors, such as hypoxia, progressive necrosis and inactivation of protein products of P53 tumor suppressor gene and Src onkogene, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). The over-expression of VEGF-D, one of isomers of VEGF, has differential effects on the uterine vasculature and these effects may facilitate VEGF-D ability to promote endometrial cancer metastasis and disease progression. Moreover, VEGF is responsible for the malfunction of a blood-brain barrier and its consequences as a malignant swelling. In gliomas also, there is an increased expression of VEGF receptors. FGF acts synergistically with VEGF in gliomas angiogenesis processes. Positive regulation by bFGF and autocrine/paracrine VEGF contributes...
to the growth and angiogenesis of brain astrocytomas, increasing the level of tumor vascularization, thus decreasing chances of survival of the patient\textsuperscript{10}.

It is suggested that tetracyclines may have clinical anti-neoplastic applications in addition to their well-established antimicrobial use as antibiotics\textsuperscript{11,12}. The antibiotics, such as tetracyclines, in particular their hemisynthetic derivatives (doxycycline, minocycline) have been reported to inhibit metalloproteinases MMP-1 and MMP-2 activity\textsuperscript{12}. \textit{In vitro} studies with MMP-2 and MMP-9 specific inhibitors, including tetracyclines, have shown to stop the process of endothelial cell invasion in collagenous stroma\textsuperscript{3,12} as an anti-angiogenic therapy in human cancer trials\textsuperscript{3,12}. Furthermore, bFGF also shows high affinity to fibrin\textsuperscript{15}. FGF binds to fibrin, influencing at the same time the structure of the produced clot and this complex stimulates proliferation of endothelial cells\textsuperscript{16,17}.

This study has been aimed to evaluate levels of selected proangiogenic factors, such as fibrinogen, VEGF and bFGF in patients with the gynecologic cancer on the first, third and sixth day of antibiotic therapy, routinely administered as a perioperative treatment, in order to find out the effectiveness of this type of therapy as an anti-angiogenic one and the pattern and direction of changes of investigated factors’ levels during the treatment. In addition, bFGF binding to \(\alpha\)-polymers and \(\gamma\)-\(\gamma\) dimers of fibrin in patients with the gynecologic cancer before and during antibiotic therapy has also been studied.

Materials and Methods

Patients

Thirteen women, aged between 50-79 yrs, with gynecologic cancer admitted to the 1\textsuperscript{st} Chair of Obstetrics and Gynecology, Medical University of Lodz, were qualified for the study. All patients gave their written consent for tissue samples to be taken for evaluation. Presented study was a part of research approved by the Institutional Ethics Board (No RNN/34/08/KE 29.01.2008). The women underwent gynecological surgery to excise tumors and received the adjuvant antibiotic therapy. In all cases, post-surgical histopathological examination of tumor tissue revealed endometroid carcinoma with the degree of differentiation: Grade-1 (3 women) and Grade-2 (10 women). Uterine carcinoma TNM (T-Tumor, N-Nodes, M-Metastasis) staging based on AJCC Cancer Staging Manual (7th ed) was as follows: \(\text{pT1bNX}\) in 5 patients, \(\text{pT1aNX}\) in 5 patients and \(\text{pT1aN0}\), \(\text{pT2N0}\), \(\text{pT1aN}\) in the remaining cases.

The examined patients were subjected to antibiotic intravenous therapy (clindamycin 3 × 600 mg with metronidazole 2 × 0.5 mg per day and/or cephalosporin 1 × 1.0 g per day). Above medicaments were administered as a perioperative prophylactics, according to regulatory directives in the department. The second control group consisted of 10 healthy women, who were under surveillance in the department. Blood samples were collected from each woman before surgery and drug application, then on the first, third and sixth day of treatment and subsequently refrigerated at -70°C for further examination. Pharmacotherapy was applied on the day of surgery, which was also the first day of treatment.

Biochemical parameters

In both investigated groups, basic biochemical blood tests, such as hematocrit (HCT), hemoglobin (HGB), white blood cell count (WBC), platelets (PLT), mean platelet volume (MPV), red blood cell (RBC) aggregation, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), sodium, potassium and total plasma proteins were performed before and at the end of treatment. Reference values are given in Table 1. Fibrinogen, bFGF and VEGF levels were also investigated.

Fibrinogen determination

To determine the fibrinogen concentration, citrate plasma (1:9) was used. The study was performed using an ACL Advance system with the PT-Fibrinogen Recombinant Kit 0020005000 (Instrumentation Laboratory Company Lexington USA) according to Clauss method\textsuperscript{18}.

bFGF and VEGF determination

bFGF and VEGF concentrations were evaluated in serum using the ELISA method (Quantikine bFGF Elisa and Quantikine Human VEGF, R&D Systems, Abingdon, UK). A standard curve was calculated in the concentration range 10-640 pg/ml for bFGF and 31,2-2000 pg/ml for VEGF. bFGF and VEGF concentrations were interpreted from the regression equation \(y = 0.0021x + 0.0154 (R^2 = 0.9913)\) and \(y = 0.00074x + 0.07169 (R^2 = 0.9983)\), respectively. Measurement of bFGF level was performed by an EL800 platelet reader at a wavelength of 450 nm (Bio-Tek Instruments, Inc.)
Blood plasma fibrinogen conversion to fibrin

The same amount of 125 µl of citrate human plasma (1:9), CaCl₂ (125 mmol/l) and NaCl (154 mmol/l) was measured out into the test tubes. Samples were placed in the incubator at 37°C for 15 min. The fibrin clot was washed with phosphate buffered saline (0.01 mol/l PBS, pH 7.4) and dissolved in 50 µl of electrophoresis sample buffer with 10 µl urea (8 mol/l) for further analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fibrin control contained: 125 µl of 1.0 mg/ml human fibrinogen, type I (>95% clottable, Sigma) in 0.01 mol/l Tris-HCl, pH 7.40, was incubated with 125 µl of 25 mmol/l CaCl₂, 125 µl of 154 mol/l NaCl 10 µl of 2 ng/ml fibrin stabilizing factor (FSF, transglutaminase, EC 2.3.13, Sigma-Aldrich) in 0.01 mol/l Tris-HCl buffer, pH 7.6. Fibrin polymerization process was initiated by adding 30 µl 12 NIH (National Institutes of Health Units) u/ml of thrombin solution (bovine thrombin, BioTrombina 400, EC 3.4.21.5, Biomed, Lublin) at 37°C for 15 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed using Mini-Protean Tetra Cell (Bio-Rad, USA) device in 12.5% polyacrylamide gel under reduced conditions (SDS-PAGE). Aliquots of fibrin prepared in a sample buffer (0.0625 M Tris-HCl, pH 6.8; 4% SDS; 50% glycerol; 20% β-mercaptoethanol and 0.25% bromophenol blue) were heated for 1 min at 95°C and run on SDS-PAGE gels (10 µg/well). The separated proteins were transferred on to a nitrocellulose membrane (Amersham™ Hybond™-ECL-GE Healthcare, 0.45µm).

Western blot analysis of bFGF bound to fibrin

Nitrocellulose membranes with transferred fibrin chains were blocked by incubation with a 5% milk solution in TTBS (0.1 M Tris-HCl, pH 7.4 containing 0.9% NaCl and 0.1% Tween 20) for night in 4°C. Then, the rabbit monoclonal antibody against bFGF was used (Anti-FGF basic neutralizing antibody, purified rabbit IgG, R&G Systems, Minneapolis) at 1:100 dilution in TTBS was added and incubated with nitrocellulose membrane for night at room temperature with constant shaking. After triple washes in TTBS (15 min each), the membranes were incubated for 1 h at room temperature with a secondary antibody (goat anti-rabbit biotynylated IgG conjugated to horse radish peroxidase (HRP) (Jackson & Jakson Laboratories, USA) at 1:300 dilution and visualized using 3,3’-diaminobenzidine (DAB) reagent (Sigma-Aldrich, USA). Quantity assessment of binded protein with Image Master 1D software (Pharmacia Biotech™ PDI Inc., v.1.20) was done using Sharp JX-330 device.

Immunohistochemical analysis

To illustrate the presence of bFGF in the tissue of excised gynecologic tumors of investigated patients, immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissues samples using anti-human polyclonal antibody against FGF basic solution 1:10 (Abcam, Cambridge). Paraffin-embedded sections of specimens were routinely processed, deparaffinized and rehydrated. Slides for immunostaining were heat-treated in target retrieval solution (pH 9.0) for 20 min. Sections were then blocked for peroxidase in 0.3% H₂O₂ in methanol for 30 min and incubated with the primary antibody for 30 min at room temperature in a humidity chamber. For detection, DAKO EnVision™+/HRP kit with DAB staining and hematoxylin counterstaining was used. For negative control, the slides were stained with the omission of primary antibody. For positive control, tissues of kidney were used.

Statistical analysis

All the results obtained during evaluation were verified with different statistical methods like t-Test (p<0.01) and Shapiro-Wilk’s Test (p>0.05). All tests were performed using StatSoft Statistica 8.0 PL software.

Results

The results of basic biochemical blood parameters before and during the treatment conducted in women with cancer, as well as in healthy subjects did not vary throughout the period of observation and are presented in Table 1. Minor changes were not statistically significant. Changes in PLT and WBC values may be explained by healing processes occurred in patients after the surgery and tumor excision.

During first 72 h of antibiotic therapy, bFGF concentration significantly increased from 10.91 ± 5.83 to 24.29 ± 17.86 pg/ml and then, on the third day it started to drop from the level of 24.29 ± 17.86 pg/ml to 9.50 ± 7.76 pg/ml, which was even below control values (9.78 ± 0.88 pg/ml) on the sixth day, indicating the effectiveness of this treatment (Fig. 1a).
Table 1—Comparison of blood biochemical parameters of patients before and after the treatment

<table>
<thead>
<tr>
<th>References</th>
<th>Before</th>
<th>After</th>
<th>Δ</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10³/μl)</td>
<td>4.0–10.0</td>
<td>9.87 ± 3.67</td>
<td>10.65 ± 2.76</td>
<td>0.78</td>
</tr>
<tr>
<td>RBC (10⁶/μl)</td>
<td>4.0–5.5</td>
<td>4.18 ± 0.45</td>
<td>4.02 ± 0.38</td>
<td>-0.17</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>12.0–16.0</td>
<td>12.92 ± 1.50</td>
<td>12.22 ± 1.08</td>
<td>-0.70</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>37.0–47.0</td>
<td>36.88 ± 4.45</td>
<td>35.52 ± 3.81</td>
<td>-1.37</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>80.0–99.0</td>
<td>87.97 ± 3.87</td>
<td>88.18 ± 4.26</td>
<td>0.21</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>27.0–31.0</td>
<td>30.85 ± 1.44</td>
<td>30.28 ± 1.47</td>
<td>-0.57</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.0–6.0</td>
<td>35.10 ± 0.93</td>
<td>34.51 ± 1.34</td>
<td>-0.59</td>
</tr>
<tr>
<td>PLT (10³/μl)</td>
<td>150.0–400.0</td>
<td>273.62 ± 63.56</td>
<td>289.46 ± 96.37</td>
<td>15.84</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.3–8.3</td>
<td>6.77 ± 0.72</td>
<td>6.15 ± 0.69</td>
<td>-0.63</td>
</tr>
<tr>
<td>Na (mmol/l)</td>
<td>135.0–145.0</td>
<td>139.69 ± 2.02</td>
<td>140.08 ± 2.81</td>
<td>0.38</td>
</tr>
<tr>
<td>K (mmol/l)</td>
<td>3.8–5.1</td>
<td>4.17 ± 0.48</td>
<td>3.79 ± 0.45</td>
<td>-0.38</td>
</tr>
</tbody>
</table>

HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PLT, platelets; RBC, red blood cell; RDW, red cell distribution width; WBC, white blood cell count.

Fig. 1—bFGF (a), VEGF (b), fibrinogen (c) levels before and during the treatment
VEGF concentration slightly lowered in the first 24 h of therapy (it dropped from 695.47 ± 438.13 to 569.65 ± 444.71 pg/ml), but on the third day of treatment, it increased (647.47 ± 462.80) to finally gain the level of 788.45 ± 433.44 pg/ml on the sixth day of observation. In comparison to control group, the level of VEGF values during whole period of investigation was 2.5-times higher. It seemed that antibiotics administration decreased bFGF level and merely influenced the level of VEGF, which elevated on the sixth day of therapy (Fig. 1b). All above data were statistically significant.

Fibrinogen concentration was found to be above the control values and continuously increased during first 3 days of therapy (from 346.43 ± 127.15 to 574.10 ± 130.21 pg/ml) (Fig. 1c). However, on the sixth day of treatment, similarly to bFGF, decreasing tendency was observed (it dropped to 491.92 ± 139.66 pg/ml at the end of an observation period). Nevertheless, the p values for t-test were higher than 0.05 and always less than 0.05 for Shapiro-Wilk test, indicating that they were not statistically significant.

In cancer patients, increased level of α-polymers and γ-γ dimers were detected (Fig. 2a-d). After antibiotic administration, amount of α-polymers increased on the third day of therapy and was statistically significant. Electrophoretic separation of fibrin on the third day of treatment is shown on Fig. 2d. Effectiveness of the antibiotic therapy was emphasized on the sixth day of observation, when there was a drop of α-polymers and γ-γ dimers levels.

Western blot analysis revealed bFGF ability to bind with the γ-γ dimers of the fibrin network. Comparing to healthy subjects, the degree of this binding before the treatment was higher (Fig. 3; Control vs. Day 0). However, bFGF did not bind with the α-polymers fraction, β and γ chains. In women with gynecologic cancer, the degree of bFGF binding with γ-γ dimers formation increased, but at the end of treatment decreasing tendency was observed.

Presence of bFGF in cancer was confirmed by immunohistochemical analysis of paraffin-embedded tissue slides using the anti-bFGF polyclonal antibodies. Figure 4 shows a positive cytoplasmatic reaction, indicating bFGF presence in an evaluated tissue.

![Fig. 2—SDS-PAGE separation of fibrin](image1)

![Fig. 3—bFGF detection in fibrin structure based on Western blot technique in control subjects, patients before surgery (Day 0) and 3 days after (The trial of 9 cancer patients during the treatment (lanes 2-10) and control fibrin (line 1))](image2)

![Fig. 4—Cytoplasmic immunohistochemical reaction in endometrial carcinoma with anti-bFGF polyclonal antibody (abcam) (Hematoxylin counterstain, magnification ×100)](image3)
Discussion

It is well-documented that the neoplastic processes are connected with thrombo-embolic complications. Hypercoagulability observed in cancer patients might be related with increased activity of thrombin, leading to conversion of fibrinogen into fibrin. The level of fibrin network in various types of cancer (e.g. breast, brain, lung and prostate cancer) is found to be relatively high. Fibrin also possesses an ability to induce tissue factor (TF) expression, causing new vessel formation by intensifying VEGF synthesis and increasing expression of proangiogenic interleukin 8 (IL-8)^20. In cancerogenesis studies^21,22, it is reported that there are fibrin structure changes and increase in platelet aggregation and fibrinogen level. Moreover, there is also an increased expression of proangiogenic factors (i.e. bFGF and VEGF)^23,26. Our study confirmed statistically significant increase of bFGF, as well as VEGF levels in cancer patients, as compared to healthy women. The level of fibrinogen was also significantly increased in sick women. Furthermore, the pattern of fibrinogen concentration during the treatment period was similar to bFGF (Fig. 1a vs. Fig. 1c).

In heart neoplasia with a characteristic sudden growth and high vascularization, FGF has been reported to play an important role in the process of angiogenesis and proliferative activity of the studied cells. The correlation between vessels density (measured by immunologic methods with endothelial cells markers) and bFGF expression is noticed in heart malignancy^24; using immunohistochemistry staining methods, the significant amount of FGF in cardiac myxoma has been indicated. Using the same method, we also confirmed the presence of bFGF in neoplastic tissue of gynecologic patients. Cytoplasmic overexpression of bFGF protein was detected in neoplastic glands in endometrial carcinoma (Fig. 4).

Results of our study showed that the VEGF and fibrinogen levels were significantly higher in patients with gynecologic cancer, as compared to healthy women. However, the difference in bFGF levels between control and cancer groups of patients was almost only statistical at the beginning of this experiment (Fig. 1a; Day 0). In cancer patients, the increase of α-polymers and γ-γ dimers concentration in fibrin network was observed. Nevertheless, bFGF was found to bind only to γ-γ dimers chains.

Earlier studies have indicated greater biochemical activity of bFGF, VEGF and IL-1β binding to fibrinogen and fibrin^27,28; however, binding of aFGF and IL1α to these proteins is not observed^28. Moreover, the specificity and high affinity of VEGF binding to fibrinogen and fibrin is reported^27. In our investigation, we evaluated the degree and specificity of bFGF binding to particular fibrin fractions under the antibiotic therapy in gynecologic cancer patients.

Western blot technique revealed the binding between bFGF and γ-γ dimers in the fibrin fractions (Fig. 3). However, the binding between bFGF and α-polymers, β and γ chains of fibrin was not confirmed, despite their presence in polyacrylamide gel (Fig. 2). The electrophoretic separation was performed using of 2-mercaptoethanol, which has the ability of breaking bonds between protein fractions (particularly -S-S-bridges)^29. The binding of bFGF to γ-γ dimers chains suggested that it was highly specific.

Anti-angiogenic activity of the antibiotic therapy in cancer patients has not been widely described in the literature. On the basis of our present study in a clinical trial of gynecologic patients, selected antibiotic therapy applied as a routine perioperative treatment appeared to have a positive influence on the pattern of selected cytokines’ levels throughout the treatment. The decrease of bFGF and fibrinogen levels seemed to confirm the effectiveness of the antibiotic therapy, which may be also considered as an anti-angiogenic one. We also observed increased α-polymers and γ-γ dimers levels in cancer patients and specific binding of bFGF to γ-γ dimers of fibrin network under the antibiotic therapy. The highest levels of analyzed parameters (except VEGF) were observed on the third day of observation, which was a day of crucial changes during the treatment. However, further studies related to the combined antibiotic therapy that can influence proangiogenic factors levels in gynecologic malignancies are required to develop a better treatment strategy for these patients.

Acknowledgements

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