Angiogenesis and Bcl-2 protein expression in patients with endometrial carcinoma

A. MAZUREK, P. PIERZYŃSKI, W. NIKLIŃSKA, L. CHYCZEWSKI, T. LAUDAŃSKI

Department of Pathophysiology of Pregnancy, Medical Academy of Białystok, 15-276 Białystok, Poland, e-mail: laudan@cksr.ac.bialystok.pl; Department of Histology and Embryology, Medical Academy of Białystok, Department of Molecular Biology, Medical Academy of Białystok, Białystok, Poland

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Considering the particular importance of angiogenesis and tumor suppressor genes expression in solid tumors, angiogenesis and Bcl-2 protein expression were evaluated in order to specify their role in the biology of endometrial carcinoma.

Clinical material comprised of 66 patients (postmenopausal, aged 52 to 76 years) with endometrial adenocarcinoma. For evaluation of angiogenesis immunohistochemical method was applied using DAKO EPOS Anti-Human Von Willebrand Factor/HRP antibodies. Morphometric method was applied to count angiogenic points (microvessels + single endothelial cells), using a light microscope with morphometric appliance. Angiogenic points density (APD) was defined as the density of AP per square mm. Immunohistochemical staining for Bcl-2 cytosomic protein expression was performed using MoAb124 (dilution 1:80, Dako A/S, Denmark) monoclonal antibodies. The percentage of 10% positive cells was considered as Bcl-2 positive tissue expression.

Positive cytoplasmic reaction of Bcl-2 was observed in 51.3% of patients with Stage I endometrial cancer, and in 23.8% and 0% of patients with II and III FIGO stage, respectively. No relationship between Bcl-2 cytoplasmatic expression and tumor grade was found. However, an inverse correlation between cytoplasmatic expression of Bcl-2 and FIGO stage was observed. The APD (angiogenic points density) was increasing with the clinical (FIGO) stage of endometrial cancer, but it was not observed in the case of tumor histologic grade.

Bcl-2 expression and angiogenesis may be a useful parameter in evaluation of the biology of endometrial adenocarcinoma as the study conducted showed the influence of Bcl-2 protein expression on angiogenesis.

Key words: Angiogenesis, endometrial cancer, Bcl-2.

Advances in molecular biology have provided clues to the pathogenesis of cancer and have shown the involvement of oncogene activation and tumor-suppressor gene inactivation. Recent evidence suggests that the genetic regulation of apoptosis is also of critical importance during tumorigenesis and that oncogenes and tumor-suppressor genes can regulate the apoptotic rate, or the susceptibility of cells to undergo apoptosis [24, 41].

During the last decade a number of known oncogenes and tumor-suppressor genes have been shown to be altered in endometrial carcinoma [5, 8, 29, 30]. Bcl-2 is the first member of a family of genes involved in the regulation of the apoptosis process. Bcl-2 protein inhibits apoptosis, reduces the requirements for growth factors and thereby extends the survival of cells.

The role of Bcl-2 in malignancy has been best described in follicular lymphomas in which the chromosomal translocation t(14;18) (q32;q21) results in juxtaposition of the Bcl-2 protooncogene and the immunoglobulin heavy chain gene [39]. In contrast, transregulatory mechanisms appear to be responsible for high levels of Bcl-2 protein expression that occurs in many different types of solid tumors, including endometrial carcinomas. The pattern and significance of Bcl-2 in endometrial carcinoma is uncertain. Several independent studies indicated hormonally dependent mechan-
ism for Bcl-2 expression in endometrium. Some studies suggest that Bcl-2 is expressed in cytoplasm in proliferative phase and down-regulated in the secretory phase [13, 21, 26, 30, 35]. CHIENG et al [8] however, found invariable expression of Bcl-2 in atypical hyperplasia and carcinoma of the endometrium.

Angiogenesis, the process of division and migration of endothelial cells, resulting in formation of new blood vessels occurs in many pathologies, both malignant – as tumor development, and benign – as arthritis or diabetic retinopathy. Physiologically it is found during fetal development, as well as in female reproductive tract, wound healing, and inflammation. Angiogenesis as a crucial step of tumorigenesis has been shown to influence the outcome of lung and breast carcinomas [9, 19].

Additionally, studies of different solid tumors, including endometrial carcinoma indicated that increased microvesSEL density within tumors was associated with metastatic potential [10, 12, 31, 34, 38, 39].

In this study we simultaneously evaluated Bcl-2 protein expression and angiogenesis and tried to correlate the findings with the clinicopathological features of endometrial carcinoma.

Material and methods

Patients. Clinical material comprised a series of 66 patients (post-menopausal, aged 52 to 76 years) with endometrial adenocarcinoma of endometroid cell type. Patients were operated by total abdominal hysterectomy with bilateral salpingo-oophorectomy at the Institute of Obstetrics and Gynecology of the Medical University of Bialystok, Poland, between years 1997 and 1999. None of the patients received preoperative pelvic irradiation. Histological typing and grading of the endometrial tumors (G-1, G-2, G-3) as well as myometrial invasion were assessed using standard criteria, on hematoxylin-eosin sections. At the surgery, FIGO clinical stage of the disease was determined. The hysterectomy specimens were evaluated for the type of carcinoma and tumor differentiation grade. Thirty nine cases were of I FIGO stage of disease, 21 and 6 patients were classified as the II and III clinical stage of the disease, respectively. Tumor differentiation grade G-1 was found in 13 cases, stages G-2 and G-3 were found in 39 and 14 cases, respectively.

Evaluation of Bcl-2 tissue protein expression (see Fig. 1 and 2). Immunohistochemical staining for Bcl-2 cytosomic protein expression in the endometrial carcinoma primary tumors was performed using MoAb124 (dilution 1:80, Dako A/S, Denmark) monoclonal antibodies. Staining was performed using indirect immunoperoxidase technique [7, 11, 22]. Sections were dewaxed and endogenous peroxidase was quenched by 30 min incubation in 0.6% H$_2$O$_2$ in methanol. Sections were rehydrated and microwaved in citrate buffer (pH 6.0) for 3x3 min. Afterwards, sections were incubated with applied antibodies overnight at 4°C. After that tissues were treated with the ABC technique using the ABC kit (Dako A/S, Denmark). Diaminobenzidine was used as a chromogen to develop immunoperoxidase reactions, sections were counterstained with hematoxylin. The same procedures were performed on the slices prepared from lymph node as a positive control. Normal nonspecific rabbit immunoglobulin-G was used as a negative control and 10% and more positive cells was considered as a Bcl-2 positive tissue expression.

Evaluation of angiogenesis (see Fig. 3). For evaluation of angiogenesis immunohistochemical and morphometric methods were applied as reported in previous publication [23]. Briefly, prepared slices were incubated with DAKO EPOS Anti-Human Von Willebrand Factor/HRP antibodies, AEC (3-amino-9-ethyl-carbazole) was used as chromagen. Negative controls were performed with DAKO EPOS Immunoglobulin/HRP. Both vessels and endothelial cells were counted under 100x magnification on a light microscope equipped with computerized morphometric appliance using PC computer and LUCIA-NIKON software. The areas of the highest endothelial cell density were chosen (so called “hot spots”). Angiogenic points (AP) were defined as microvessels and single endothelial cells including closed capillaries. Angiogenic points density (APD) was defined as the density of AP per square mm.

Statistical analysis. To check the influence of histopathologic tumor grade and FIGO stage of the disease on the APD (angiogenic points’ density) two-factor analysis of variance (ANOVA) was applied. To compare the mean value of APD for different values of Bcl-2 t-test was used. In order to fulfill the model assumptions, the logarithm transformation of the APD values was done. Fisher’s exact test was used to analyze relations of FIGO stage of disease and tumor’s grade to Bcl-2. The trend occurrence was checked by Cochran-Armitage test for trends. All the tests were two-sided. The 0.05 significance level was applied. Analysis was performed using SAS/STAT software.

Results

The immunohistochemical study of the Bcl-2 protein revealed that 25 (38%) of 66 samples had a positive staining in 10% or more of tumor cells. Bcl-2 overexpression occurred in 51.3% of patients with stage I endometrial cancer, 23.8% and 0% with II and III stage, respectively (Tab. 1). Cochran-Armitage test for trend revealed the occurrence of statistically significant trend (p < 0.004) of decreasing the percentage of Bcl-2 positive tumors with the stage of disease.

With respect to tumor grade, Bcl-2 tissue overexpression was observed in 58.3% (7/12) of G-1 grade, in 33.3% (13/39)
Table 1. Correlation between Bcl-2 tissue expression and stage and grade of disease

<table>
<thead>
<tr>
<th>FIGO stage of disease</th>
<th>Bcl-2 overexpression</th>
<th>p-value*</th>
<th>p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia, Ib, Ic</td>
<td>20/39</td>
<td>0.015*</td>
<td>0.046**</td>
</tr>
<tr>
<td>IIa, Iib</td>
<td>5/21</td>
<td>0.206*</td>
<td></td>
</tr>
<tr>
<td>IIIc</td>
<td>0/6</td>
<td>0.318</td>
<td>0.212</td>
</tr>
</tbody>
</table>

Table 2. Correlation between angiogenic points density (APD) and stage and grade of the disease

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>APD (AP/mm²)</th>
<th>Log APD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO stage of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia, Ib, Ic</td>
<td>39</td>
<td>155.8±74.6</td>
<td>4.9±0.43</td>
</tr>
<tr>
<td>IIa, Iib</td>
<td>21</td>
<td>195.9±56.2</td>
<td>5.2±0.29</td>
</tr>
<tr>
<td>IIIa</td>
<td>286.9±94.8</td>
<td>5.6±0.29</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade of disease</th>
<th>APD (AP/mm²)</th>
<th>Log APD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>12</td>
<td>137.8±47.9</td>
<td>4.86±0.38</td>
</tr>
<tr>
<td>G2</td>
<td>39</td>
<td>200.7±88.4</td>
<td>5.2±0.45</td>
</tr>
<tr>
<td>G3</td>
<td>15</td>
<td>162.1±60.2</td>
<td>5.01±0.4</td>
</tr>
</tbody>
</table>

Table 3. Relationship between Bcl-2 expression and angiogenic points density (APD)

<table>
<thead>
<tr>
<th>Bcl-2 status</th>
<th>No. of cases</th>
<th>APD (AP/mm²)</th>
<th>Log APD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>41</td>
<td>140.1±49.5</td>
<td>4.88±0.35</td>
<td>0.0009**</td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>205.1±85.4</td>
<td>5.23±0.42</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The preoperative identification of high-risk endometrial cancer cases is currently suboptimal. Although clinical staging does correlate with the outcome, assessment of the disease spread is inaccurate in approximately one third of patients [22]. Histologic profile and subtype are characteristics of the primary tumor basing on the preoperative endometrial biopsy. These traditional prognostic factors might enhance the diagnostic accuracy in detection of patients who are at risk for recurrence [27].

Bcl-2 is the first member of genes family involved in regulation of the process of apoptosis. Little is known about the role of Bcl-2 expression in the genesis or malignant progression of endometrial carcinoma. The relationship between Bcl-2 and grade of differentiation in some types of cancer, including endometrial carcinoma, has been discussed in a number of studies [5, 29, 30, 33].

Our study demonstrated the overexpression of Bcl-2 in 51.3% of patients with stage I endometrial cancer, and in 23.8% and 0% of patients with II and III FIGO stage, respectively.

Neman et al [25] found that Bcl-2 expression in endometrial carcinoma was less intense than in complex hyperplasia. It is controversial whether Bcl-2 expression is correlated with the tumor grade. Chan et al [5] found an inverse correlation between the grade of endometrial carcinoma and Bcl-2 expression, the Bcl-2 expression decreased as the tumor became less differentiated. Henderson et al [16] found no correlation between Bcl-2 and histologic grade of endometrial carcinoma.

In our study, we did not find any relationship between Bcl-2 cytoplasmic expression and tumor differentiation grade. However, we found an inverse correlation between cytoplasmic expression of Bcl-2 and FIGO stage of the disease.

Angiogenesis is of crucial importance for the tumor growth and development of metastases [15]. The intensity of tumor vascularisation is thought to reflect the angiogenic activity generated by the neoplastic cells or the supporting stroma [18, 38]. The biology of tumor angiogenesis and its clinical significance has been studied in a variety of gynecologic malignancies. Several studies have been reported in ovarian carcinoma [17, 32] and in cervical carcinoma [4, 20, 36, 40]. Guidi et al [14] have demonstrated that in early cervical lesions the MVD (microvessel density) was significantly increased both in invasive carcinoma and high grade intraepithelial lesions, as compared with low grade intraepithelial lesions in cases of positive Bcl-2 cytoplasmic expression the mean value of angiogenic points density was significantly (p < 0.02) lower (140.1 ± 49.5 AP/mm²) than in Bcl-2 negative tumors (205.1 ± 85.4 AP/mm²) (Tab. 3).
Figure 1. Bcl-2 expression in well differentiated endometrial carcinoma showing 100% of positive cancer cells. MoAb124 (Sako A/S, Denmark) monoclonal antibodies were used. Original magnification x400.

Figure 2. All cancer cells are Bcl-2 negative in the case of well differentiated endometrial carcinoma. Original magnification x400.

Figure 3. Many blood vessels (hot spot) in the stroma of moderately differentiated endometrial carcinoma. Vessels are marked through the endothelial cell visualization using DAKO EPOS Anti-Human Von Willebrand Factor/HRP antibodies. Original magnification x200.

pithelial lesions and benign squamous epithelium. However, an inverse correlation for MVD for patients with cervical carcinoma has been reported [20, 28]. In our study the APD was increasing with the clinical (FIGO) stage of endometrial cancer, ranging from 155.8 ± 74.6 SP/mm² in I clinical stage to 286.9 ± 94.8 AP/mm² in the II stage of disease. Such a phenomenon was not observed in case of the tumor differentiation grade, the maximal angiogenic points density was found in medium differentiated (G-2) tumors – reaching 200.7 ± 60.2 AP/mm².

Abulafia et al [38] reported increased angiogenesis of complex endometrial hyperplasia as compared to controls of simple hyperplasia. The angiogenic capability of complex hyperplasia was comparable to endometrial carcinoma of FIGO stage Ia. An increased angiogenesis was found in cases of invasive (stages Ib and Ic) endometrial carcinoma as compared to the complex hyperplasia or stage Ia endometrial carcinoma. In our study we correlated tumor angiogenesis and Bcl-2 expression and we found that APD value was significantly lower in Bcl-2 positive tumors.

Boldrini et al [3] observed an inverse correlation between Bcl-2 protein expression and vascular count in cases of non-small lung cancer. Carmaliet et al [6] found that reduced oxygen tension leads to suppression of Bcl-2 gene and to increase of VEGF levels and angiogenesis. On the other hand, in breast carcinoma cell line in hypoxic conditions, Biroccio et al [2] found an increase of VEGF protein secretion in cases of Bcl-2 positive cells. Since VEGF is considered as potent angiogenesis activator, these findings may link Bcl-2 expression and angiogenesis.

In conclusion, our study showed statistically significant trend of decreasing the percentage of Bcl-2 positive tumors with the stage of disease. Angiogenic points density varied significantly between groups of FIGO stage. In cases of positive Bcl-2 cytoplasmic expression mean value of angiogenic points density was significantly lower than in Bcl-2 negative tumors.

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[31] Sieira A, Llovetes R, Castellanaque X, Moreno L, Garcia-


