Histoenzymatic and immunohistochemical study during antiangiogenetic treatment in breast cancer cells

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Abstract
Breast cancer is the second most frequent cause of death by cancer. In Romania, its incidence is around 4200 new cases per year and the mortality is around 2500 cases per year. During the past few years a great number of anti-neoplastic therapies with higher specificity and efficiency were developed. Monitoring the effects of these polychemotherapies is of great importance together with the study of their influence on cellular metabolism. The purpose of the study was to establish the changes in the cellular energy metabolism and apoptotic potential in the anti-neoplastic therapy with bevacizumab. The results obtained show that the apoptotic potential of malignant cells increases significantly during the anti-angiogenic treatment, with reduction of tumor vasculature and that the mitochondrial apoptotic pathway is activated by releasing cytochrome c from the mitochondrial inter-membrane space.

Keywords: angiogenesis, bevacizumab, breast carcinoma.

Introduction
Breast cancer is the most common neoplasm in women, representing 32% of all cancers and 18% of deaths from cancer. In the European Union the incidence of breast cancer is 105 and mortality is 40/100 000 women.

Some authors consider that the elucidation of metabolic mechanism in cancer will set the basis for effective solutions in cancer treatment [1, 2].

In this study, we aimed to follow the cellular metabolic activity with the help of some histoenzymatic techniques and the changes produced under anti-angiogenic treatment in breast cancer cells. The apoptotic potential of cells was also assessed using the immunohistochemical analysis for determining the cytochrome c release from the mitochondrial inter-membrane space [3, 4].

Along with exploring the therapeutic potential of apoptosis it is necessary to discover the mechanism and molecules involved. Pharmacological manipulation of apoptosis offers new possibilities for treatment [5–7].

Histoenzymatic analysis, which was included in the experimental protocol, helped determining the development of the different metabolic processes in cells and tissues, allowing an enzymatic activity to be highlighted at the cellular site where it actually takes place. The discovery of the regulating mechanism of these metabolic pathways would stock or deliver important information for the diagnosis as well as for the anti-neoplastic therapy.

We assessed the changes in the cellular energetic metabolism and apoptotic potential in anti-neoplastic therapy with bevacizumab. This drug was recently introduced in Romania, in 2007, and its action on the energetic metabolism has not yet been studied.

Anti-angiogenic drugs block the vascular endothelial growth factors, preventing the formation of new vessels. This is a modern strategy for anti-cancer therapy. Vascular endothelial growth factor (VEGF) is characterized by the most powerful stimulation of angiogenesis because of its specificity almost exclusively on endothelial cells. Monoclonal antibody, bevacizumab binds vascular endothelial growth factor (VEGF), a key factor of vasculogenesis and angiogenesis, and thus inhibits VEGF binding to its receptors FLT-1 (VEGFR-1) and KDR (VEGF-2) from surface endothelial cells [8, 9]. Neutralization of biological activity of VEGF causes regression of tumor vessels, normalizes the remaining vasculature and inhibits the formation of new tumor vessels, thereby inhibiting tumor growth. The study we have conducted followed the effects of anticancer therapy based on histoenzymatic and immunohistochemical methods.

Materials and Methods
In the study were included patients with breast
cancer who underwent primary treatment with partial response or stationary disease. Patients with complete response or disease progression were not included. In patients with complete response to primary treatment, other treatment is not justified and in patients with disease progression, another anti-cancer treatment should be started.

In AI group, 21 patients (mean age 56.50 ± 10.60 years) were enrolled who did not receive anti-angiogenic treatment, due to objective causes (heart disease, vascular disease, renal disease, etc.) or subjective ones (refusal). This group represents the control group in terms of response to anti-angiogenic treatment.

In the AII group, 22 patients (mean age 53.75 ± 12.77 years) were included who underwent anti-angiogenic treatment with bevacizumab (CNAS protocols) after primary anti-cancer treatment. This group represents the study group (Figure 1).

Each patient signed the informed consent form. Mammary tumor tissue samples were used, from AI and AII group, which were obtained by biopsy following the surgical procedures at the County Hospital of Oradea (Romania), between 2007–2010. The samples were sectioned using a cryotome, then assessed using histoenzymatic methods, to highlight the activity of the following enzymes:

- Adenosine triphosphatase (ATP-ase): Represent a class of enzymes, which are active in the mitochondrial membrane and cell membrane, and catalyse the decomposition of ATP into ADP and free P-ion. This reaction releases energy. ATP-ase activity is more intense as it decomposes cytosolic ATP. The highlighting of this enzyme was performed using the Wachstein and Meissel method. Tissue areas with enzyme activity appear brown-black.

- Lactate dehydrogenase (LDH): Is a NAD(P)-dependent cytoplasmic enzyme which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. Highlighting this enzyme was performed using the Hess–Scarpelli–Pearse method. Tissue areas with enzyme activity are dark blue.

- Succinate dehydrogenase (SDH): Is a component of mitochondrial enzyme complex II, which plays a key role in Krebs cycle. Highlighting this enzyme was performed using the Nachlas–Tsou–Souza–Chang–Seligman method. Tissue areas with enzyme activity appear blue [10–12].

Histoenzymatic analyses, which were included in the experimental protocol, helped in determining the development of the different metabolic processes in cells and tissues, allowing an enzymatic activity to be marked out at the cellular site it actually occurs.

For the immunohistochemical highlighting of cytosolic cytochrome c, the cytochrome c Ab-2 (clone 7H82CR) kit – mouse monoclonal antibody – was used [13].

### Results

#### The study of adenosine-triphosphatase activity (ATP-ase)

In the AI control group, ATP-ase shows a heterogeneous distribution both at the baseline and after six months of treatment. It was observed that intensely stained areas alternate with weakly stained areas (Figures 2 and 3). In the AII study group there was a decrease of ATP-ase activity after six months of anti-angiogenic treatment (Figures 4 and 5).

#### The study of lactate-dehydrogenase activity (LDH)

At baseline (month number 0), both in AI group and AII group, the LDH activity is intense. In the AI group, the activity of this enzyme remains similar after six months, compared with the AII group in which there is an enhanced activity of LDH (Figures 6 and 7).

#### The study of succinate-dehydrogenase (SDH)

Enzyme activity areas appear blue. Enzyme activity gives indications about the Krebs cycle. The succinate-dehydrogenase activity in the control group AI is unchanged during the six months of study.

In the AII study group the baseline histoenzymatic stain analysis shows a balanced activity of the SDH enzyme. During the sixth months there is a decrease in mitochondrial enzyme activity in response to hypoxic conditions that occur in which there is a decrease of mitochondrial enzyme activity and especially of Krebs cycle (Figures 8 and 9).

#### Immunohistochemical study of apoptosis using the technique for cytosolic cytochrome c

The baseline immunostaining for cytochrome c is
weak with numerous negative areas both in group AI and AII. After six months, in group AII there is an intense positive immunoreactivity for cytochrome c (Figures 10 and 11).

Figure 2 – AI group (month 0). Invasive ductal carcinoma; ATP activity; areas marked with arrows show intense enzyme activity (×100).

Figure 3 – AI group (month 6); Invasive ductal carcinoma; ATP activity; areas marked with arrows show intense enzyme activity (×100).

Figure 4 – AII group (month 0); Invasive ductal carcinoma; ATP activity; areas marked with arrows show intense enzyme activity (×100).

Figure 5 – AII group (month 6); Invasive ductal carcinoma; ATP activity; areas marked with arrows show intense enzyme activity (×100).

Figure 6 – Group AII (month 0); LDH activity; areas marked with arrows show intense enzyme activity (×100).

Figure 7 – Group AII (month 6); LDH activity; areas marked with arrows show intense enzyme activity (×100).
Figure 8 – Group AII (month 0); SDH activity; areas marked with arrows show intense enzyme activity (×100).

Figure 9 – Group AII (month 6); LDH activity; areas marked with arrows show intense enzyme activity (×100).

Figure 10 – AII group (month 0); Rare items with positive immunoreactivity for cytochrome c; cells marked with arrows show a cytoplasm with positive immunoreactivity for cytochrome c (×400).

Figure 11 – AII group (month 6); Positive intense immunoreactivity for cytochrome c; areas marked with arrows show intense positive immunoreactivity for cytochrome c (×400).

Discussion

The results describe a complex metabolic image of the malignant cells before and after the initiation of the anti-neoplastic treatment.

Before the anti-angiogenic treatment the HE staining shows cellular features characteristic of malignancies, anisocytosis, nucleus/cytoplasm ratio changes, tachy- chromatic nuclei, atypical mitoses.

The presence of intratumoral vascularity was revealed, supplying oxygen and nutrients for tumor cells, but together with an intense glycolytic metabolism, demonstrated by the excessive LDH activity.

LDH activity was studied in many tumor cell types and the studies found a significant increase of the glycolytic enzyme [14, 15]. Our studies confirm these results, demonstrating the existence of aerobic glycolytic metabolism by the presence of intense vascularization. At the mitochondrial level, the metabolic activity is low, the tricarboxylic acid cycle is carried out with lower intensity but mitochondria show no loss of membrane integrity, cytochrome c is not released in the cytosol, and the cells are not involved in the apoptotic process.

After the anti-angiogenic treatment, there is a significant decrease in mitochondrial enzyme activity in response to the emergence of hypoxic conditions in which the mitochondrial enzyme activity and the Krebs cycle in particular, reduce the ATP activity and amplify the glycolytic pathway of the glucose metabolism.

The apoptotic potential of malignant cells increases significantly during the anti-angiogenic treatment, the mitochondrial apoptotic pathway is activated by releasing cytochrome c from the mitochondrial intermembrane space in the cytoplasm, highlighted by the immunohistochemical technique.

Conclusions

Studies have shown that before the anti-angiogenic treatment there is a very intense glycolytic metabolism, highlighted by the excessive activity of LDH. ATP
synthesis is achieved mainly through the glycolytic pathway. The mitochondrial activity had low intensity, but mitochondria show unimpaired membrane integrity, cytochrome $c$ is not released in the cytosol, the cells are not involved in apoptosis, ATP is synthesized in increased quantities. After anti-angiogenic treatment the rate of glycolysis increases, due to installed hypoxia, reduced ATP synthesis, activated mitochondrial pathway of apoptosis by releasing cytochrome $c$ from the mitochondrial inter-membrane space.

Reduction in tumor blood vessels with subsequent decreased oxygenation leads to a deeper state of hypoxia.

In this state, the mitochondrial invaginations are distended, the mitochondrial ATP synthesis decreases, and thus the glycolytic pathway of the glucose metabolism is activated. Due to the accumulation of lactic acid, the pH decreases which results in the inhibition of cellular enzymes and leads to cell autolysis. Hypoxia induces the activation of HIF (hypoxia inducing factor), which stimulates angiogenesis by regulating VEGF, which explains the utility of bevacizumab monoclonal antibody administration.

It was been demonstrated that the apoptotic potential of malignant cells increases significantly during anti-angiogenetic treatment, with reduction of tumor vasculature. The mitochondrial apoptotic pathway is activated by releasing cytochrome $c$ from the mitochondrial inter-membrane space.

References


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