Monitoring the effects of treatment in colon cancer cells using immunohistochemical and histoenzymatic techniques

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Abstract

Background: Monitoring the effects of treatment in malignant diseases is very important in study of the influence on the cell metabolism. Energy production in cancer cells is abnormally dependent on aerobic glycolysis. In addition to the dependency on glycolysis, cancer cells have other atypical metabolic characteristics. The purpose of the present study is to evaluation and analysis of the colon cancer cells under anti-angiogenic treatment, to establish the changes in the cellular energy metabolism and apoptotic potential. Anti-angiogenic drugs block the vascular endothelial growth factors, preventing the formation of new vessels. Materials and Methods: We use immunohistochemical analysis of cytochrome c release and histoenzymatic analysis of adenosine triphosphatase (ATP-ase), succinate dehydrogenase (SDH), lactate dehydrogenase (LDH) enzymes. Colorectal tumor tissue samples were obtained by biopsy following the surgical procedures at the County Clinical Hospital of Oradea (Romania). Results: The obtained results show that the apoptotic potential of malignant cells increases during the anti-angiogenic treatment, in the same time the rate of glycolysis increases, due to installed hypoxia and reduced ATP synthesis. Our results have been confirmed by international studies too. Conclusions: It was been demonstrated that the apoptotic potential of malignant cells increases significantly during anti-angiogenic treatment. There is growing evidence that cancer’s ‘Achilles’ heel’ is tumor cell metabolism.

Keywords: colon cancer, cellular energy metabolism, apoptosis.

Introduction

Colorectal cancer is the third most common cancer in the world, with nearly 1.4 million new cases diagnosed in 2012 and is the second most frequent cause of cancer death in men. In Romania, its incidence is around 18.55/100 000. Approximately 95% of colorectal cancers are adenocarcinomas. Other types of cancer that can occur here include mucinous carcinomas and adenosquamous carcinomas [1].

A great number of anti-neoplastic therapies with higher specificity and efficiency were developed during the past few years.

The metabolic properties of cancer cells diverge significantly from those of normal cells. Energy production in cancer cells is abnormally dependent on aerobic glycolysis. In addition to the dependency on glycolysis, cancer cells have other atypical metabolic characteristics [2–4].

In 1956, Warburg observed that the rate of glycolysis was abnormally high in cancer cells [5–7].

Energy metabolism of malignant cells is the aim of recent research on the treatment of neoplasia. The study of energetic metabolism is now considered an unexplored area of the attempts of improvement and treatment of malignant neoplasia [8, 9].

Although the molecular mechanisms that define the Warburg effect are not yet fully understood, the increased glycolysis observed in cancer cells. In addition to the dependency on glycolysis, cancer cells exhibit other metabolic characteristics such as increased fatty acid synthesis and glutamine metabolism [2, 3, 10, 11].

Alterations in respiratory activity appear to be a general feature of malignant cells. The process of formation of new vessels from existing vessels, angiogenesis, plays an important role in nutrient intake and metabolic support in tumor cells [8, 12, 13].

Research has shown that modulating the activity of respiratory chain can induce differentiation or death of cancer cells. This will become possible introduction of anticancer mechanisms by modulating cellular respiration [8, 14]. The discovery of these regulatory mechanisms and metabolic pathways altered mitochondria and its role in the malignant cells dedifferentiation will provide important information for anti-cancer diagnosis and treatment [9, 14, 15].

In healthy cells, cytochrome c (cyt c) is located in the mitochondrial intermembrane, intercristae spaces, where it functions as an electron shuttle in the respiratory chain. Several reports have indicated that chemotherapy agents trigger apoptosis by inducing release of cytochrome c from mitochondria [14, 16–19].

The essential hallmarks of cancer are intertwined with an altered cancer cell-intrinsic metabolism, either consequent or as a cause. As an example, the resistance of cancer mitochondria against apoptosis-associated permeabilisation and the altered contribution of these organelles to metabolism are closely related [2, 3, 8, 20].

Adenozine triphosphatases (ATP-ases), lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) are enzymes directly involved in cellular energy metabolism. ATP-ases represent a class of enzymes, which are active...
in the mitochondrial membrane and cell membrane, and catalyze the decomposition of ATP into ADP (adenosine diphosphate) and free phosphate (P) ion. LDH is a NAD(P)-dependent [nicotinamide adenine dinucleotide (phosphate)] cytoplasmic enzyme, which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. SDH is a component of mitochondrial enzyme complex II, which plays a key role in Krebs cycle [21–26].

Bevacizumab is a recombinant humanized monoclonal antibody, is an anti-angiogenic drug, directed against the vascular endothelial growth factor (VEGF), preventing the formation of new vessels. VEGF is characterized by the most powerful stimulation of angiogenesis because of its specificity almost exclusively on endothelial cells. Monoclonal antibody, Bevacizumab binds vascular VEGF, a key factor of vasculogenesis and angiogenesis, and thus inhibits VEGF binding to its receptors FLT-1 (Fms-related tyrosine kinase-1, VEGFR-1) and KDR (kinase insert domain receptor, VEGF-2) from surface endothelial cells [27–29]. 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 [30].

The purpose of this study was to establish the changes in the cellular energy metabolism and apoptotic potential in the anti-angiogenic therapy. In the past, we have studied these issues in breast cancer cells [26], but we wanted expansion the study on colorectal cancer too.

Materials and Methods

In the BI Group, 24 patients were included (mean age 57.55±9.80 years), of which 14 males and 10 females. They enrolled patients who after completing primary oncology treatment did not receive anti-angiogenic treatment, due to objective causes (renal disease, heart disease, vascular disease, etc.) or subjective ones (refusal). This group represents the study group. Treatment was continued until disease progression. Each patient signed the informed consent form. This study is accomplished over a period of six months of starting anti-angiogenic treatment.

Colorectal tumor tissue samples were obtained by biopsy following the surgical procedures at the County Clinical Hospital of Oradea (Romania).

Immunohistochemical identification of cytosolic cytochrome c (cyt c)

An immunohistochemical analysis was performed on 4 μm-thick sections prepared from formalin-fixed paraffin-embedded tissue by using an automated immunostainer (Bechmark XT, Ventana Medical Systems Inc., Tucson, AZ, USA). Immunohistochemical assays were performed on a Ventana Benchmark XT automated staining instrument according to the manufacturer’s instructions. Slides were deparaffinized using EZprep solution (Ventana Medical Systems, Inc.) at 90°C, and all reagents and incubation times were chosen as directed on antibody package inserts. Slides were developed using the OmniMap DAB (3,3'-diaminobenzidine) detection kit (Ventana Medical Systems, Inc.) and counterstained with Hematoxylin [31–33].

For immunohistochemical identification of cytosolic cytochrome c, sections were incubated with the mouse monoclonal antibody, clone 7H 8 2CR, Ab-2 kit, according to the manufacturer’s instructions [33].

Histoenzymatic analysis of the activities of energy metabolism enzymes

The histoenzymatic analysis was performed on 4 μm-thick sections using a cryotome, then highlights the activity of the following enzymes: ATP-ase, SDH, and LDH. ATP-ase is active in the mitochondrial membrane and cell membrane, and catalyze 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 [26]. The highlighting of this enzyme was performed using the Wachstein and Meissel method. Tissue areas with enzyme activity appear brown-black.

SDH is a component of mitochondrial enzyme complex II, which plays a key role in Krebs cycle [6, 8, 26]. The highlighting of this enzyme was performed using the Nachlas–Tsou–de Souza–Chang–Seligman method. Tissue areas with enzyme activity appear blue [22, 26, 34, 35].

LDH is a NAD(P)-dependent cytoplasmic enzyme. It converts pyruvate, the final product of glycolysis to lactate [23, 26]. Highlighting this enzyme was performed using the Hess–Scarpelli–Pearse method. Tissue areas with enzyme activity are dark blue.

Results were evaluated by accepted semiquantitative method for percentage expression [36]. In 100× microscopy images for histoenzymatic analysis and 200× microscopy images immunohistochemical analysis were randomly chosen 10 different fields.

The semiquantitative evaluation was performed as follows:

- “-”: absence of enzyme activity or immunoreactivity;
- “+/-”: rare elements of enzyme activity or with immunoreactivity;
- “+”: positive enzyme activity or immunoreactivity;
- “++”: intense positive enzyme activity or immunoreactivity [36, 37].

Figure 1 – Distribution of patients in the BI control Group and BII study Group.

This group represents the study group. Treatment was continued until disease progression. Each patient signed
Results

Immunohistochemical study of apoptosis using the immunohistochemical identification technique of cytosolic cyt c

Before anti-angiogenic treatment, we found more areas with negative immunoreactivity for cyt c in both BI and BII Groups (Figure 2).

In BI Group, the percentage of areas with absence of immunoreactivity and rare elements of immunoreactivity are increased in both months 0 and 6.

In BII Group, after six months of anti-angiogenic treatment, there is an intense positive immunoreactivity for cyt c, the apoptotic potential of malignant cells increases significantly, as seen in Figures 3 and 4. The obtained results can be observed in Figure 5, in a case of colon adenocarcinoma after six months of anti-antigenic treatment [18, 19, 38].

The study of adenosine triphosphatase (ATP-ase) activity

In the BII study Group, after six months of anti-angiogenic treatment (Figures 6 and 7) there was a decrease of ATP-ase activity.

Takes place a massive reduction of very intense enzyme activity from 34% to only 5% (Figure 8). ATP-ase activity decrease is lower than previously studied in breast cancer cells [26].
In the same time, in the 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. ATP-ase activity is very intense in areas with malignant transformation. The results for the ATP-ase activity in the BI control Group are similar to those obtained in breast cancer cells [4, 26].

**The study of SDH activity**

SDH enzyme activity gives indications about the Krebs cycle. The SDH activity in the BI control Group is unchanged during the six months of study. In the BII study Group, can be seen changes, by reducing the SDH enzyme activity after anti-angiogenic therapy, demonstrating reduced mitochondrial activity, and especially of Krebs cycle (Figures 9–11).

**The study of LDH activity**

LDH is located in the cytoplasm; it is an oxidoreductase participating in anaerobic glycolysis. At baseline, in both BI and BII Groups, the LDH activity is intense (Figure 12). In the BI Group, the activity of this enzyme remains similar after six months, compared with the BII study Group in which there is an enhanced activity of LDH (Figures 13 and 14).
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Discussion

Colorectal cancer causes 608,000 deaths per year, despite the correct treatment applied for this disease, 30% to 50% of these tumors present recurrences [39]. Our study was performed on 22 patients, BII Group. They underwent anti-angiogenic treatment after primary anticancer treatment. BI Group represents the control group in terms of response to anti-angiogenic treatment.

The purpose of the present study was to evaluation and analysis of the colon cancer cells energy metabolism under anti-angiogenic treatment, to establish the changes in the cellular energy metabolism and apoptotic potential. We used immunohistochemical and histoenzymatic analysis.

The obtained results describe a complex image about tumor cell metabolism and apoptotic potential in colon adenocarcinoma cells under and without anti-angiogenic treatment. Mitochondrial cyt c has been found to have dual functions in controlling both cellular energetic metabolism and apoptosis. Through interaction with apoptotic protease activating factors, cyt c can initiate the activation cascade of caspases once it is released into the cytosol [16, 38, 40]. A mitochondrially derived redox signal is simultaneously generated because of mitochondrial cyt c release [16, 40]. In the following study group, after six months of anti-angiogenic treatment, there is an intense positive immunoreactivity for cyt c, the apoptotic potential of malignant cells increases significantly, 78.8% with analyzed microscopy fields shows positive and intense positive immunoreactivity. These results show efficiency of anti-angiogenic treatment in activation of apoptotic pathways.

The histoenzymatic analysis was performed for study cellular energy metabolism. The analysis highlights the activity of the following enzymes: ATP-ase, SDH, and LDH, thus tracking the activity of these enzymes shows us the metabolic activity of the studied cells.

The results obtained prior to initiating the study confirm that energy production in cancer cells is abnormally dependent on glycolysis [2, 3, 4, 23, 40]. ATP-ase activity results are similar to those achieved in breast cancer cells. The enzymatic activity was significantly reduced in the study group, as in the previous study [26].

After anti-angiogenic treatment, SDH enzyme activity is reducing, demonstrating reduced mitochondrial and Krebs cycle activity. In the same time, increased LDH activity, an oxidoreductase participating in anaerobic glycolysis, suggests the activation of the glycolysis, by converting the lactate into pyruvate, with NADH coenzyme. Increased LDH and SDH activity is less intense in colon cancer cells than breast cancer cells [20, 24, 26].

In general, we can affirm that these results are similar to those obtained in breast cancer cells [26], but highlighted changes in activity of the studied enzymes are less evident comparing the two groups, the BI control Group and BII study Group.

Several reports have indicated that chemotherapeutic agents trigger apoptosis by inducing release of cyt c from mitochondria [14, 16, 17, 18, 21, 40]. The dependence of tumor cells on glycolysis for ATP generation offers a rationale for therapeutic strategies aimed at selective inhibition of the glycolytic pathway [21, 23, 41].

Our results show that prior to initiating the study the mitochondria shows no loss of membrane integrity, cyt c is not released in the cytosol, and the cells are not involved in the apoptotic process, in the same time the cellular energy metabolism is accelerated, shown by ATP-ase and SDH increasing activity. After six months of anti-angiogenic treatment, there is an intense positive immunoreactivity for cytosolic cyt c, the apoptotic potential of malignant cells increase significantly. The activity of ATP-ase and SDH enzymes is reducing with the increase of LDH enzyme activity, showing us the inactivated cellular energy metabolism, depending from mitochondria activity.

The resistance of cancer cells to treatment is often associated with flaws in their apoptotic program. Successful elimination of tumor cells, therefore, largely depends on the ability of anticancer treatment to stimulate silent or suppressed apoptotic pathways. Mitochondria are promising targets for such an approach [8, 41].

Mitochondria physiology in cancer cells is linked to the Warburg effect. Besides, its central role in apoptosis makes this organelle a promising “dual hit target” to selectively eliminate tumor cells [23, 40].
Conclusions

The study of cellular energy metabolism considered now as an unexplored area of the attempts of improvement and treatment of malignant neoplasia. Due to this, we evaluated and analyzed the energy metabolism of the colon cancer cells under anti-angiogenic treatment. In the study group with decrease ATP-ase and SDH activity, takes place an increase in LDH activity and an intense positive immunoreactivity for cytosolic cyt c, after six months of anti-angiogenic treatment. At the mitochondrial level, the metabolic activity is low; the Krebs cycle is carried out with lower intensity. By correlating these results, we found that the mitochondrial ATP synthesis decreases, and thus the glycolytic pathway of the glucose metabolism is activated. The mitochondrial apoptotic pathway is activated by releasing cyt c. We contributed in the demonstration that the apoptotic potential of malignant cells increases significantly during anti-angiogenic treatment. There is growing evidence that cancer’s “Achilles’ heel” is tumor cell metabolism.

Conflict of interests

The authors declare that they have no conflict of interests.

References

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