



THE DUAL ROLE OF ACTIVATED SPECIES OF OXYGEN IN ANGIOGENESIS

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Abstract. Lately, the expanding accumulation of scientific data has indicated/demonstrated that malignant tumor growth depends on local angiogenesis. This occurs as a result of a process induced by a subgroup of transformed cancer cells which adopt an angiogenic phenotype, resulting in a new local balance of proangiogenic and antiangiogenic factors. The purpose of our work is to explore the hypothesis that activated species of oxygen play an important role in the pathways that stimulate the angiogenesis favouring tumour growth. The study was performed in vivo, on rats with experimental RS-1 tumours treated with bevacizumabum. We made a dynamic analysis of biochemical parameters of oxydative stress (the level of lipid peroxydes, thylic groups, total antioxydants); apoptosis was measured by flow-cytometry in tumoral and normal hepatic tissues. We registered a decrease in the oxidative status during the antiangiogenic treatment and a significant increase of apoptosis (from 9.12% to 20.14%) in the malignant cells. These data support the hypothesis that activated species of oxygen can initiate the proangiogenic phenotype transformation. In this kind of oxidative circumstances targeted antiangiogenic drugs can reverse the process and induce tumour cells apoptosis, resulting in clinical benefits for cancer patients.

Key words: angiogenesis, bevacizumabum, activated oxygen species, experimental tumours, apoptosis

Introduction

Angiogenesis is a complex of coordinated cellular and molecular processes having as a result new vessels formation, departing from the previous vascular net [1]. This is a two step process, with a phase of activation, which results in new vessels formation, followed by a phase of maturation, with vessel stabilization [2].

The activation phase implies basal membrane destruction around the old vessel, followed by migration and proliferation of endothelial cells. A cord of endothelial cells is formed, which becomes tunnelled and connects to another vessel. In these phases the vascular endothelial growth factor (VEGF) and angiopoetin 2 (ANG2) are activated.

The maturation phase of the new vessel makes possible the stabilization by basal membrane reconstruction and perivascular cells (pericytes) recruitment. In this phase several process intervene/act: platelet derived growth factor (PDGF), angiopoetin 1 (ANG1), transforming growth factor beta (TGFbeta)

and bone morphogenesis protein (BMP) [3].

Most of the tests that measure in vitro angiogenesis investigate only the activation phase (for example protease secretion) offering an incomplete view of the process. Even so these tests led, during the last years, to the discovery of a number of molecules that are essential for the angiogenesis process[4]. The hypoxic signalization pathway which induces, through HIF (hypoxia inducible factor), the expression of proangiogenic factors VEGF and ANG2 is essential for neoangiogenesis[5][6]. Reactive oxygen species (ROS) are known as mediators of angiogenesis and as having a role in metastatic potential of tumour cells[7].

By modulating the activity of different enzymes and critical transcription factors oxidative stress can activate a series of intracellular signalization pathways[8]. In such a scenario, increase transcription factors activated by ROS will translate from cytoplasm to the nucleus binding to the promoter regions of specific genes[9]. As a result, activation of such pathways with consecutive gene expression modulation can heavily determine cells; activities and course of action (cytokine production, proliferation or apoptosis). The balance between ROS production and antioxidant cellular defence, activation of oxidative stress related to signalising pathways with gene transcription products appearance will determine

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if a cell or group of cells exposed to ROS increase will survive or die [10].

Bevacizumabum is a relatively new monoclonal antibody, which acts as an anticancer drug by binding to VEGF receptors and blocking them. This prevents the binding of VEGF and blocks the activation phase of angiogenesis. The formation of new vessels is prevented [11]. As a result tumour growth is stopped. Vascular depletion results also in tumour cell death. Normal cells are not or less affected. As a consequence the safety profile is much more favourable than those of the cytotoxic drugs.

Our objective was to identify and characterize the role of ROS in the cascade of angiogenesis signalization with consequent antitumor effects. We developed an experimental *in vivo* model. Angiogenesis inhibition was obtained using Bevacizumab. The resulting variation of parameters was quantified and followed during the Avastin administration.

Materials and Methods

In vivo experimental design

Our study was performed *in vivo*, on animals carrying the experimental RS-1 hepathoma. The used animals (Wistar rats) originated from the Bucharest Institute of Cancer, accredited for biologic material use from 1964 (when it was created).

In conformity with what was stipulated at Bratislava 2002 Conference "Ethics in Scientific Research" and in accordance with the Royal Society for Animal Violence Protection from UK we consider that suffering provoked to the animals during the research, as a consequence of stress reaction can influence the accuracy of scientific data. The principle of pain limiting (to reduce as much as possible the animal suffering provoked by the experiment) and that of number reduction (using the minimal number of animals necessary to attain study objectives) were implemented. Working on small rodents, the experiment design and control methods were conceived having in mind both the importance of the problem and the limits of safety. Evaluation of toxicities was based on behaviour observation/including growing, biochemical tests and also on histological and necropsy reports. Our experimental model respected the relevant legislation (the principles to be followed when working with small animals) and the recommendations of the Ethical Committee of the Institute. The animals were kept in the Institute's Biobasis, with standard conditions of feeding and hydration. They were separated in two arms, one with no treatment and the second one treated with Avastin (for 3 months, 5 days weekly, 5mg/kg/day, in normal saline solution, 0,2 ml iv). The treatment started 21 days after tumour inoculation.

Tumour implantation

The RS-1 hepathoma is a transplantable carcinoma, chemically induced in rats by 2-acetileaminofluoren. The tumour is maintained to present time by subcutaneous transplantations to Wistar albino rats; it is defined by a latency period of 25-30 days and a rate of successful transplantation of 90%. It does not

metastasise; local invasion can be observed, median survival of porters is 70-80 days. Approximately one million cells/ml were inoculated in the right flank. The tumour can be maintained also by intrahepatic inoculation. Subcutaneous and hepatic greffons are histologically identical (solid carcinoma, with mucinous areas), with a low mitotic activity and a moderate rate of apoptosis and spontaneous necrosis. It is an ovoid, neatly delimited tumour, with a smooth capsula of rose-yellow colour. Necrotic and hemorrhagic areas are alternating in its structure.

Biochemical assays

The index of lipid peroxidation was evaluated by measuring the serum malonaldehyde, the final product of lipid hidroperoxids degradation. The method is based on a new red adduct formation (MDA-TBA₂) with a maximum of absorption at 532 nm, registered on a Analytic Yena Specord 210 spectrophotometer. The solutions are prepared on site using genetic pure water from a Milli-Q device. The substances used (barbituric acid, acethical acid, trichloroacetic acid) are provided by Merck and have the required purity grade [12][13][14]. Lipid peroxides were measures in tumoral and in normal liver tissue.

Albumin thiols determination

Albumin-thiols groups were determined using the reaction with 5,5'-ditio-bis (2-nitrobenzoic) acid Ellman reactive. Reactives were prepared on site utilizing chemical compounds provided by Merck. They were measured in serum, tumour tissue and normal liver tissue by spectrophotometry.

FRAS (ferric reducing ability of serum)

The reaction measured the reduction of Fe³⁺-2,4,6-tri(2-piridil)-1,3,5-triazina (TPTZ) to a coloured product. The method which measures the combined antioxidant effect of non-enzyme antioxidants from biologic fluids is used in controlling the index of resistance to oxidative destructions. At low pH, when the above mentioned complex is reduced to Fe²⁺ by the oxidants from the probe, it results in blue coloration with λ_{max} 593nm. In this method an excess of Fe³⁺ is used while the limiting factor for the complex (and colour) formation is the antioxidant (the redactors from plasma) [16][17].

Flowcytometry measurements

Anexin V a calcium dependent anticoagulant protein has a big affinity for fosphatidilserine, binding selectively to negatively charged fospholipides. By conjugation to a fluorochrome (for example fluorescein) it can be used as a marker for apoptosis identification, together with DNA colour reaction with propidium iodure (18).

The detection of hypodiploid DNA by flow cytometry using the quantitative binding reaction of propidium iodure (the most used colorant) to DNA (as a result of cell permeabilisation) is the most common technique for apoptosis determination [19]. Apoptotic cells have a low level of DNA and can be recognised

by their position on DNA content histograms, the low G1 peak, positioned at the left of the peak representing cells from the G1 phase of the cell cycle. The absence of the hypodiploid peak is not always a proof for apoptosis absence. However, the presence of this peak cannot be considered a proof for an apoptotic population in the absence of supplementary proofs [20].

Results

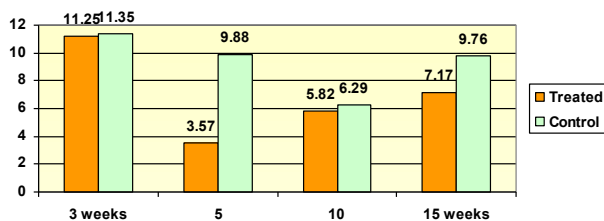


Fig. 1. Lipid peroxidation reaction in normal liver tissue

As a reference tissue we chose the normal liver tissue. On measuring one can see a significant increase of lipid peroxidation in the observation arm, a dynamic rise after a decrease to one third of the values registered at five weeks of treatment. The data suggests that the initial inhibition of the oxygen metabolism is rapid, with subsequent restoring values, even if the treatment with antiangiogenic agent continues. We suppose that this phenomenon is based on low initial concentrations of ROS, gradual elimination of hypoxia and signalization of the events necessary to neovascularization.

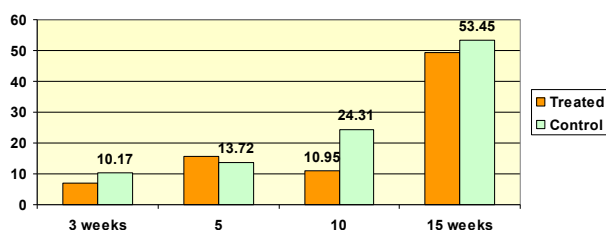


Fig. 2. Lipid peroxidation in tumoral tissue

In the tumoral tissue one can see the same profile of lipid peroxidation during the antiangiogenic treatment; the rise is more important in the observation (untreated) arm.

SH group during the anti- VEGF treatment in tumoral and liver tissue

Albumin – thiol groups were processed from the same biological materials, tumour tissue versus normal liver tissue, with the same dynamics. Identical final products of oxidative-degradative reaction appear as a result of ROS attack on sul containing proteins. It is a well known fact that tumour tissue can produce an excess of proteins that offer antioxidant protection (metaltioenins, glutation, cistheine,etc). Dynamics rises from the treatment point to the antioxidant defence mechanisms in the tumoral tissue.

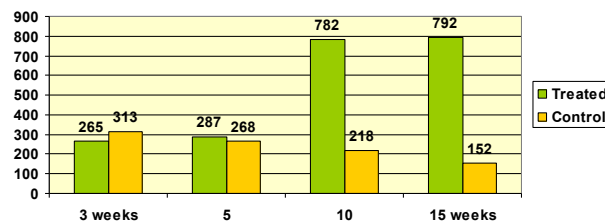


Fig. 3. The value of albuminic thiols in liver tissue

In the normal liver tissue the value differs from the tumor tissue; in the treated arm we can see lower registered values for the studied item, comparing to the observational arm.

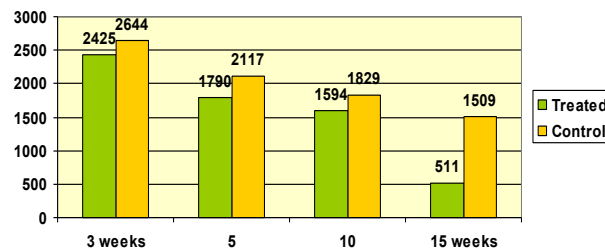


Fig. 4. The value of albuminic thiols in liver tissue

As a result of ROS, induced signalling endogen antioxidant defence systems were activated; as a result the obtained values were lower in the treatment arm (versus those in the observational arm).

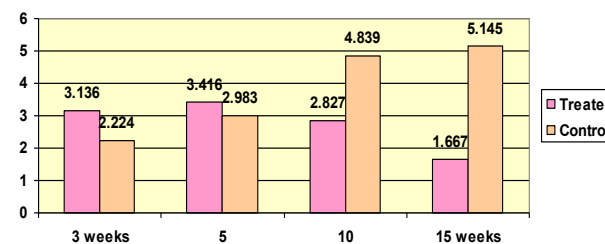


Fig. 5. Total antioxidants at the hepatic level

In the normal liver tissue the antioxidant profile is higher. We can interpret this only as a result of the activation of liver metabolism and detoxification after prolonged treatment. These mechanisms involve liver enzymes as those from the cytochrome family, which produce ROS.

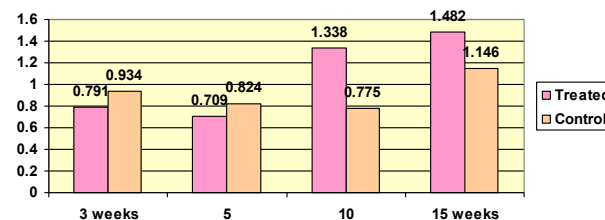


Fig. 6. Total antioxidants in tumour tissue

The obtained biochemical data suggest that bevacizumabum inhibits the production of ROS the first month of treatment in hypoxic conditions, in a dose-dependent pattern. After that the profile of apoptosis directed oxidative reactions is modified. These results are presented in the following diagrams, constructed on flow cytometry determinations.

Apoptosis determination by flow-cytometry

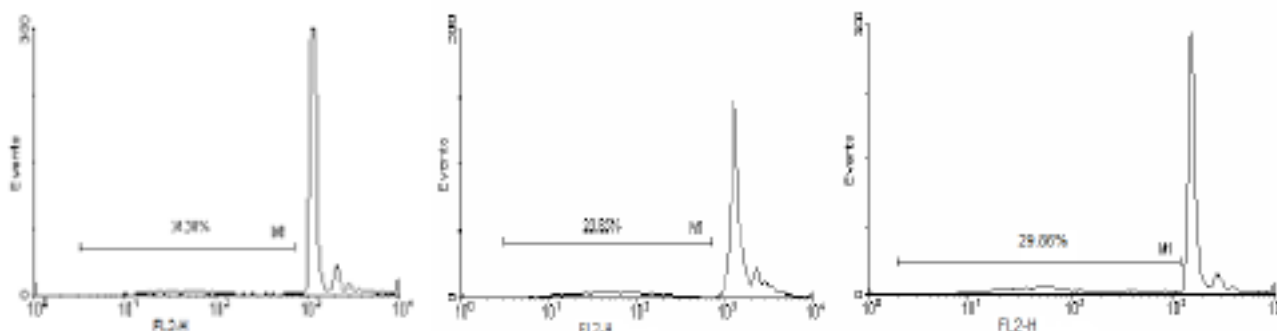


Fig. 7. The apoptosis diagrams obtained by flow-cytometry in normal liver tissue

In these diagrams one can observe a rise in apoptosis from 14,3% to 29,6% during the treatment phase. The results suggest an apoptotic treatment-induced mechanism. Data for tumour tissue follow.

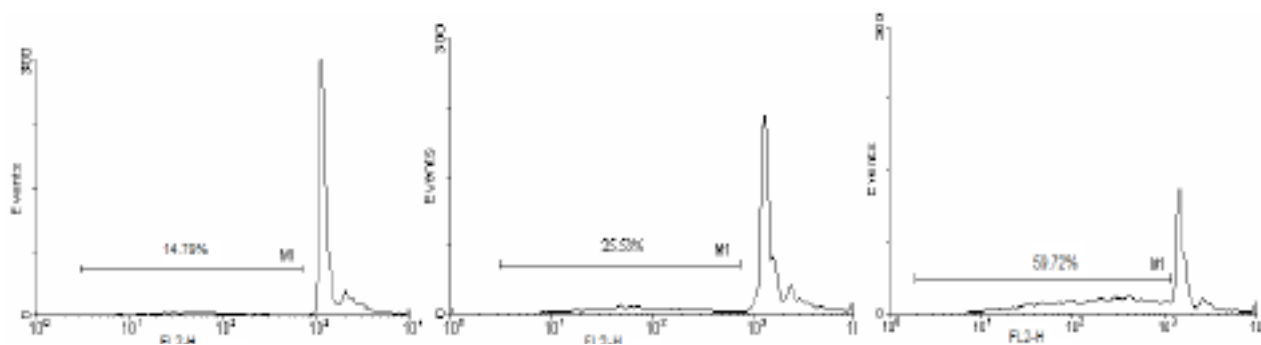


Fig. 8. The apoptosis diagrams registered during treatment in tumour tissue

They show a higher increase in apoptosis, up to 59,72% thus demonstrating the treatment's efficacy on the tumour tissue, expressed by apoptosis induction instead of angiogenesis blockage.

Conclusions

The comparison of data collected through by measurements on tissue homogenates obtained from animal carriers of experimental RS-1 hepatoma treated with bevacizumab versus data obtained in the observational, non-treated arm led to the following conclusions:

- lipid peroxidation in the tumour tissue is not significantly changed after antiangiogenic treatment

- one observes a rise of total thiols and of the level of antioxidants suggesting an activation of natural defence systems against ROS, after an oxidative stress

- treatment with the antiVEGF monoclonal antibody bevacizumab significantly changes the cell phenotype, towards an apoptotic one. After the treatment there is a significant increase of the apoptosis in the tumour tissue

- the data suggest that ROS have a dual role in this process; at lower levels, corresponding to the first exposure to avastin they have a role of signalling hypoxia. This stimulates angiogenic effects. Biochemical mechanism will be redirected to ROS production that be involved in the apoptotic cytotoxicity.

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