Therapeutics, Pharmacology and Clinical Toxicology
Vol XVIII, Number 4, December 2014
Pages: 160-163

ORIGINAL PAPER

RESULTS OBTAINED FROM THE ASSOCIATION OF BORON NEUTRON CAPTURE THERAPY WITH BEVACIZUMAB

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Abstract. The angiogenesis process represents one of the most optimised biological processes in the human body. With our experiment we aimed to explore the oxidative stress route as enabler of angiogenesis inducing signal pathways. We materialized this scenario by using a lot of experimentally induced RS-1 hepatoma cells carrier animals that we treated with an angiogenesis inhibitor and, in a later phase, with BNCT irradiation. The serum was processed by centrifugation in view of determining the oxidative stress parameters, while cells from the tumour tissue were collected with the purpose to establish apoptosis through flow-cytometry. Results obtained from flow cytometry measurements indicate the occurrence of apoptosis after a second course of Bevacizumab. After a first round of Bevacizumab, apoptosis increased from 9.12% to 20.14% but dropped to 12.05% following BNCT administration. These results highlight an expansion in the oxidative metabolism, at the level of the tumour tissue, compared to values registered after serum determination. The administration of an anti-angiogenic compound triggers lower oxygen and nutrients flow thus determining a slower lipid peroxidation reaction. Even in the context of oxygen intake limitation it turned out that the albumin thiols' value in the tumour tissue augments. Ceruloplasmin is not expressed in the murine tumour tissue. The obtained values lead to several conclusions, namely: oxygen reactive species are secondary signalling messengers for the initiation of vital biochemical reactions; when produced in small quantities they will have an active participation in the signalling cascade but when they come in large concentrations they become destructive effectors and thus can be used in the monitoring of radiation therapy.
Key words: angiogenesis, oxygen reactive species, BNCT

Introduction

It is well known that angiogenesis deploys through multiple stages and consists in the formation of new blood vessels that provide oxygen and food intake to the tumour cells.

The entire process is accompanied by a cascade series of events where chemical mediators take form, a major role being attributed to VEGF- the vascular endothelial growth factor. VEFG selectively binds to the tumour cell surface and can be quantified by immunohistochemistry methods. The latest data in literature have shown that tumour growth and progression are angiogenesis dependant[1]. Most human tumour cells stay in situ for long periods (that could take months to years) in a dormant, avascular status. During this stage the tumours contain several million cells. When a group of the tumour cells advances to the agiogenic phenotype due to a change in the local equilibrium between positive and negative regulators of angiogenesis, the tumour rapidly grows and becomes clinically detectable. An obvious question is linked to the signalling mode for the change in the local equilibrium.

It is our presumption that oxygen reactive species initiate this signalling. Put into a certain scenario oxidative stress may activate signalling metabolic pathways, modulating the activity of various enzymes and critical transcription factors. The latter migrate from the cytoplasm to the nucleolus and bind to a certain region from a specific gene promoter. Hence, this activated stress pathway may have a significant impact on the gene expression that will certainly affect the cell's viability (apoptosis, proliferation, cytokine production, etc). The balance between the production of oxygen reactive species, cellular defence mechanisms, activation of stress signalling pathways and the production of

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compounds from different genes will determine whether a cell exposed to oxidative stress lives or dies[2, 3, 4, 5].

Materials and methods

We materialized this scenario by using a lot of RS-1 experimentally induced hepatoma carrier animals that we treated with an angiogenesis inhibitor. This is a monoclonal antibody that binds to the VEGF receptor. Once it is bound it will discontinue the growth and dissemination of cells, blocking the neovascularisation process, as well. The pharmaceutical compound used by our team is Bevacizumab, a compound that has already been tested and integrated in the anti-tumour antibodies used in clinical treatment of various solid tumours. [6]

RS-1 alveolar hepatoma type used in our experiments is a chemically induced tumour obtained by mixing 2- acetylaminofluorene in the rats' food. The initially obtained tumour was kept functional by tumour grafts serial transplantation. Thus we obtained a hepatocellular cholangiocarcinoma that maintained its characteristics even after serial passages. Consequently to the subcutaneous tumour grafting, the tumour failed to produce metastases leading to a lifespan of 70-80 days in our experiment rats.

The Wistar rats were treated with bevacizumab 5 mg/kg weekly for a month after which BNCT protocol was applied.

The RS-1 hapatoma carriers received, once a week, four doses of anti-angiogenic compound, after which they were anesthetized with 1% Acepromazine- 2.5 mg/Kg and 10% Ketamine- 75mg/kg , at an average weight of 150 grams.

At this stage the animals were applied the BNCT irradiation protocol. They received i.v. BPA in 5% fructose solution at a 300 mg/kg dosage. The neutron source was a TRIGA-ACPR nuclear reactor elaborated in an enclosure provided to our team with the curtsey of our partners at the National Institute for Physics and Nuclear Engineering- Horia Hulubei – Magurele. The animals were immobilized on a plastic-based strip and introduced in the irradiation device. The time was set at 2 hours with an epithermal neutron intake of 107n/cm2. The filter collimator assembly in the spectrometry gamma prompt device was placed at the level of the reactor's radial channel. We obtained a fluency of thermal neutrons of 1.418*109 and 2.13*109 n/cm2, while the epithermal neutron's was 1.145*105 and 1.472*105 n/cm2 following BPA solution in 5% fructose. Irradiation was undertaken 3-5 hours after BPA administration.

The irradiation time was set at 120 minutes. Upon waking, the animals were hydrated with water ad libitum, transported back to the laboratory where they were sacrificed in view of collecting serum and tumour tissue, as biological material. The serum was processed by centrifugation in order to determine oxidative stress parameters, while the collection of cells from the tumour tissue aimed to establish apoptosis through flow-cytometry measurements.

Results and discussions

The results obtained by our team as a consequence of flow-cytometry assay indicated apoptosis initiation after the second administration with Avastin.

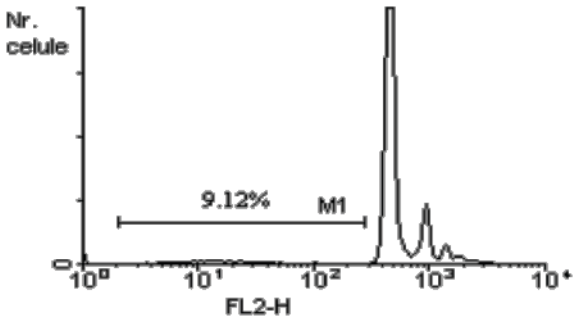

Figure 1. Apoptosis of the tumour cells prior to the administration with Bevacizumab

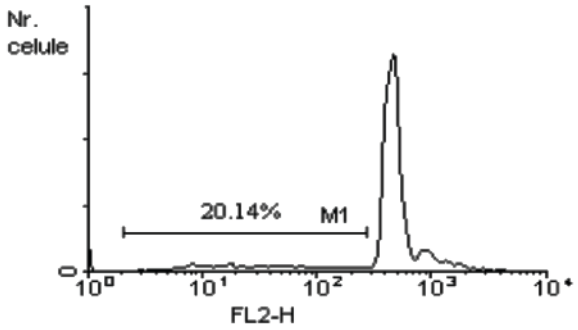

Figure 2. Apoptosis of the tumour cells after the first Bevacizumab administration. The graphic displays an increase in the white blood cells percentage consequently to the angiogenesis inhibition.

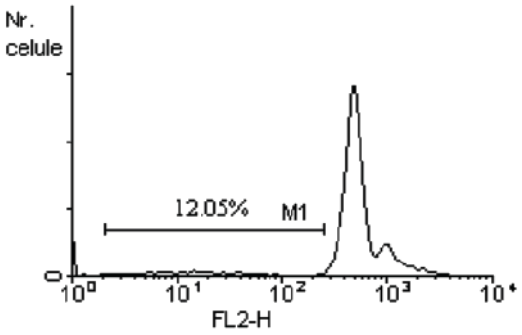

Figure 3. Apoptosis of the tumour cells after 2 administrations with Avastin and BNCT

The graphic presents the apoptosis of the tumour cells after two treatment sessions with Avastin and BNCT. Apoptosis increased from 9.12% to 20.14% after the first round of Bevacizumb but decreased to 12.5% following BNCT. The values are not cumulative this suggesting that a higher BNCT efficacy requires oxygen in site[7]. However, the values obtained are difficult to interpret considering that the used antibody-bevacizumb is a human monoclonal antibody and not a murine one. Hence, the immune reactions occurring as a response to human antigens may, as well, induce apoptosis but the contribution of the immune response versus the anti-angiogenic effect to the induction of tumour apoptosis is still difficult to quantify.

The data for the oxidative stress parameters displayed

in Table 1 present the mean values associated with the complete Avastin treatment compared to the data on BNCT irradiation and cumulative ones:

	Anti-angiogenic treatment	BNCT	Avastin +BNCT
Lipid peroxidation Normal values 0-2µmols/100 ml serum	10,38	11,67	12,16
Ceruloplasmine Normal values 80-120 U.I.	131	142	148
Albumine thiols Normal values 370-450 µmols/l	116	143	240
Total antioxydants Normal values 0,9-1.2 µmols/ml	1,08	1,23	1,89

Table I. Oxidative stress parameters detected in the serum consequently to Avastin+BNCT association.

Determinations were processed on the gross tumours, as presented in the table below.

	Anti-angiogenic treatment	BNCT	Avastin +BNCT
Lipid peroxidation	10,44	8.64	7.63
Ceruloplasmine	5	4	2
Albumine thiols	305	440	592
Total antioxydantsl	1,28	1,43	1,29

Table II. Parameters of the oxidative stress assessed in the association between Avastin + BNCT, from the gross tumour extract.

Once the hepatic detoxification systems become active peroxides expand, as an overall consequence of a xenobiotic administration- BPA. This is the reason why throughout the entire experiment all obtained values exceeded normal values. Lipid peroxidation surpasses the normal range within the serum of animals that carry tumours but also because they are produced in excessive quantity by the tumour itself, after which they are released in the circulatory flow. In a healthy liver, lipid peroxides increase subsequently to the activation of the detoxification systems- mainly cytochrome dependant whose main site is the liver. The cytochrome system is among the first affected if the liver function fails. In a liver affected by tumours, as response to their presence, sulphur proteins are excessively synthesized (glutathione metallothionein), with antioxidant reactions and capture of excess peroxides[8, 9, 10].

Although the main site of the ceruloplasmine synthesis is found is the liver, its antioxidant copper oxidase action (measured experimentally) takes place in the peripheral areas (in the serum) and not in the liver itself, this explaining the increased values in the serum and the decreased ones in tissues.

As a defence reaction in tissues, sulphur proteins are excessively synthesized. Their degradation due to the albumin thiols production measured during this experiment reached an increased level, compared to values registered in the peripheral areas[11, 12].

The outcomes reflect an expansion of the oxidative metabolism in the presence of tumours versus the values identified at the serum determination. The tumour relies on stronger and more rapid series of antioxidant defence mechanisms that provide an explanation for its resistance to different types of treatment. Administering the anti-angiogenic compound implies an intake in the oxygen flow and lower levels of nutrients that trigger a decrease in the lipid peroxidation reaction. After assessing the results we can safely assume that the primary targets of oxygen reactive species are the lipids, the proteins, on the other hand, being attacked subsequently to the initiation of chain reactions. The value of albumin thiols in the tumour tissues amplifies even if the oxygen intake is limited. This can partially be explained by the biochemical tumour protection that contains the protein's synthesis, namely sulphate and glutathione, metallothionein which as a consequence of the oxidative attack releases free and antioxidant reactions initiating SH groups. Ceruloplasmin is not expressed in the murine tumour tissue.

Conclusions

The values recorded during the experiment lead to the conclusion that oxygen reactive species are secondary signalling messengers for the initiation of vital biochemical reactions. Also, when they are produced in low concentration they will engage actively in the signalling cascade but if produced in higher concentrations they become destructive effectors and can be used when monitoring the efficacy of radiation treatments. It is very important to establish, as clearly as possible, their level as they can set off a series of endogenous antioxidant defence mechanisms that diminish the effects of antineoplastic treatments.

In what concerns the association between bevacizumab and BNCT therapy, a conclusion is far from being drawn, but apparently the relative oxygen deficit in the tumour, obtained by inhibiting angiogenesis can be detrimental.

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