Expression of soluble biomarkers associated to cell proliferation, angiogenesis and oxidative stress in rats bearing Walker tumours

Received for publication, January, 25, 2019
Accepted, March, 18, 2019

DANIELA GLAVAN¹, CAMELIA MIA HOTNOG²*, VALENTINA NEGOITA¹, MIRELA MIHAILA², LORELEI IRINA BRASOVEANU²**, MARIA IULIANA GRUIA¹,
¹ “Prof. Dr. Alexandru Trestiorean” Institute of Oncology, Bucharest, Romania
²“Stefan S. Nicolau” Institute of Virology, Center of Immunology, Bucharest, Romania
*Principal co-author
**Address for correspondence to: Lorelei I. Brasoveanu, e-mail: luli_brasoveanu@yahoo.com; “Stefan S. Nicolau” Institute of Virology, Center of Immunology, 285 Mihai Bravu Ave, S3, 030304, Bucharest, Romania, phone/ fax: +40-21-3241471

Abstract
Carcinogenesis is a process that occurs in several stages where distinct changes occur, both at molecular and cellular levels, and involves three stages: tumour initiation, promotion, and progression. Proliferation is an important stage in the development and progression of cancer, and includes alterations of the expression and/or activity of the cell cycle related proteins, activation of multiple signal transduction pathways. Part of the survival strategy of these cells may be manifested by changes in cellular metabolism. After the onset of tumours, growth and metastasis can be sustained by overproduction of hormones (in hormone dependent cancers), by promoting angiogenesis, triggering autophagy. Tumours are biologically characterized in order to emphasize their proliferative, invasive and metastatic abilities. Aberrant glycosylation is often considered a “cancer fingerprint”, playing a fundamental role in tumour development and progression. Resistance to treatment, escape from host immune system control, tumour invasion and angiogenesis, metastasis are closely related to aberrant sialilation of glycoproteins and glycolipids. The malignant phenotype is associated with the combined action of several exo-and endopeptidases, like cathepsin D. Oxidative stress refers to the imbalance between free radicals that form the pro-oxidant system and endogenous antioxidants, and the accumulation of reactive species in cancer cells plays an important role in the initiation and evolution of this disease. The aim of our study was the development of an experimental in vivo model, based on Walker 256 tumour bearing rats, for concomitant evaluation of the expression of soluble biomarkers associated with tumour progression, angiogenesis and oxidative stress. Since Walker 256 is a carcinosarcoma tumour, the implemented experimental model might be of great use in the oncology clinic to improve diagnostic, prognostic, metastatic risk assessment and treatment monitoring.

Keywords: cathepsin D, sialic acid, oxidative stress, Walker 256 carcinosarcoma

1. Introduction
Carcinogenesis is a process that occurs in several stages where distinct changes occur, both at molecular and cellular levels, and involves three stages: tumour initiation, promotion, and progression. Proliferation is an important stage in the development and progression of cancer, and includes alterations of the expression and/or activity of the cell cycle related proteins, activation of multiple signal transduction pathways (WEBER & al. [1]). Initial stages of tumour development are associated with a fibrinogenic response, and the development of a hypoxic environment that promotes the survival and proliferation of cancer stem cells. Part of the survival strategy of these cells may be manifested by changes in cellular metabolism (JEMAL & al. [2]). After the onset of tumours, growth and metastasis can be sustained by overproduction of hormones (in hormone dependent cancers), by promoting angiogenesis,
triggering autophagy. Tumours are biologically characterized in order to emophasize their proliferative, invasive and metastatic abilities. Aberrant glycosylation is often considered a "cancer fingerprint", playing a fundamental role in tumour development and progression. Thus, glycans play an important role in signaling cancer cells, dissociating and invasion of tumour cells, cell-matrix interactions, and angiogenesis, metastasis and immune modulation (LAZESCU & al. [3]; XIAOLU & al. [4]). Resistance to treatment, escape from host immune system control, tumour invasion and angiogenesis, metastasis are closely related to aberrant sialilation of glycoproteins and glycolipids (TEOH & al. [5]; KOHNZ & al. [6]; CUI & al. [7]). Basal membrane disruption is a feature of malignancy. The malignant phenotype is associated with the combined action of several exo- and endopeptidases, like cathepsin D, that are involved in an enzymatic cascade which facilitates extracellular matrix degradation, an important stage in the invasion of tumour cells (DIAN & al. [8]; PRANIOL & al. [9]; PRANIOL & al. [10]). Oxidative stress refers to the imbalance between free radicals that form the pro-oxidant system and endogenous antioxidants, either due to the exaggerated action of the oxidants or because of the reduction in endogenous fighting ability. The role of reactive oxygen species (ROS) in cancer has been extensively investigated in recent years, as the accumulation of reactive species in cancer cells plays an important role in the initiation and evolution of this disease (GURER-ORHAN & al. [11]; GRUIA & al. [12]; GRIGORESCU & al. [13]). ROS regulates each stage of tumour development, including transformation, survival, proliferation, invasion, metastasis and angiogenesis. Oxidative stress can cause angiogenesis in carcinomas because oxygen radicals increase production of angiogenic factors (REUTER & al. [14]; PANIERI [15]). Therefore, the aim of our study was the development of an experimental in vivo model, based on Walker 256 tumour bearing rats, for parallel evaluation of the expression of soluble biomarkers associated with tumour progression, angiogenesis and oxidative stress. Since Walker 256 is a carcinosarcoma tumour, the implemented experimental model might be of great use in the oncology clinic to improve diagnostic, prognostic, metastatic risk assessment and treatment monitoring.

2. Materials and methods
2.1. Experimental animal model and tumour graft: Studies were performed on 60 healthy Wistar albino rats weighing 200 g on average. They were inoculated with 1x10^6 tumour cells/ml in the right flank. The tumour volume was measured with a vernier caliper and calculated according to the formula: \( V = a \times b^2 \times 0.52 \), where \( a \) and \( b \) represent the maximum and the minimum tumour diameters, respectively, and 0.52 = skin thickness (mm). Rats were sacrificed after 7, 10, 14, 21, 28 and 35 days for blood and tumour collection. Male Wistar albino rats weighing 200-220 g (2 months old) were used. The animals were housed in standard boxes with standard laboratory diet and water ad libitum. The protocol was approved by the institutional animal ethics committee constituted for the purpose. The in vivo experimental models were elaborated on legally based decisions about principles of good laboratory practice.
2.2. Evaluation of soluble cathepsin D: Briefly, cathepsin D concentration used is similar to that described by Capony (CAPONY & al. [16]). To 100 µl of sodium formiate buffer (1M, pH=3.5) there were added 100 µl of 4% hemoglobin and 150 µl serum or pure cathepsin D solution.
2.3. **Evaluation of soluble sialic acid** was performed with a colorimetric method described by Kattermann. Dosage of free or bound sialic acid is based on periodic oxidation, resulting in β- formylpyruvic acid formations, it reacts with two thiobarbituric acid molecules and forms a pink chromophore with maximum absorbance at 549 nm.

2.4. **Evaluation of lipo-peroxides in sera:** The lipid peroxidation index (PX) was determined by the malonylaldehyde assay (KANDAR & al. [17]; GRUIA & al. [12]).

2.5. **Evaluation of total thiol groups (TSH) in sera:** The total thiol groups (TSH) were assayed as described by Schosinski, using Ellman reagent (ELMANN & al. [18]; JANERO & al. [19]; GRUIA & al. [12]).

2.6. **Evaluation of total antioxidants (AO) in sera:** The total antioxidants (AO) were evaluated using the method of Benzie & Strain.

2.7. **Statistical Analysis:** All biochemical determinations were done in triplicate and are expressed as mean values. was performed by using Student t’ and GraphPad tests; p values < 0.05 were considered statistically significant.

3. **Results and discussion**

3.1. **Characterization of Walker 256 carcinosarcoma**

Walker 256 carcinosarcoma is a standard tumour used in the preclinical screening of antitumour substances, as well as in various experimental models of chemo-radio-immunotherapy. Also, the tumour is widely used as an experimental model for inducing cachexia in the rat. It is a rapidly transplanting rat carcinoma that spontaneously appeared in the mammary gland of a pregnant rat (reported in 1928). In the early stage of development, the tumour has a carcinomatous (well vascularized) appearance, so that in a more advanced growth phase it has a sarcoma appearance. Microscopically, in some cases, only the carcinomatous or sarcomatous aspect can be emphasized, while in other cases, the two aspects can coexist within the same tissue (LEWIS & al. [20]).

![Macroscopical and microscopical aspects of Walker 256 carcinosarcoma](image)

The percentage of subcutaneous tumour graft grafting is 80-90%, with seasonal variations; the tumour metastasizes rarely, and the loco-regional lymph node invasion occurs in the terminal tumour growth phase when tumour necrosis phenomena are accentuated. Also, some studies indicate the presence of hepatic and pulmonary metastases at the final stage of the disease.

The control group consisted of 10 healthy rats, while other 50 rats were inoculated with 1,000,000 tumour cells / mL (Walker's tumoural cell tumour - ascitic form) in the right flank. Cell viability was determined with 0.1% Tripan Blue in TFS (saline phosphate buffer).
Ten rats were sacrificed at 7, 14, 21, 28 and 35 days from the graft, at which time the primary tumour was assessed and biological samples were collected: tumour tissue, to obtain smears and blood samples, in order to obtain the serum used in subsequent dosages. Thus, following the slaughter of 10 rats at weekly inoculation, induction of tumours was found. The fastest increase occurs in the first week, when the tumour volume reaches 1.1 cm³. The tumour volume continues to increase, after 14 days reaching 2.1 cm³, after 21 days - 3.8 cm³, after 4 weeks it grows up to 4.5 cm³, and after 5 weeks it reaches 4.8 cm³. Microscopy analysis showed variable cells in form and size, predominantly large and ovoid, cytoplasm abundant, vesicular, moderately basophilic, and irregular nuclei in form and size, with lax chromatid and partially visible nucleoli (Figure 1).

3.2. Evaluation of soluble cathepsin D and sialic acid in Walker 256 bearing tumour rats

Proteases play an important role in invasion and metastasis. Studies have shown that certain proteinase groups are secreted by tumour cells and respond to the proteolytic cascade during the invasion. Cathepsin D is an ubiquitously expressed lysosomal protease. In all cell types examined to date, it is synthesized as a mannose-6-phosphate modified 52-kD glycoprotein. This transient intermediate glycoprotein is transported from the Golgi apparatus through target lysosomal receptors.

![Figure 2. Soluble biomarkers in sera from Walker 256 bearing tumour rats](image)

Table 1: Serum levels of cathepsin D and sialic acid in Walker 256 tumour-bearing rats

<table>
<thead>
<tr>
<th>Days after tumour inoculation</th>
<th>Cathepsin D (nM)</th>
<th>Acid sialic (mM)</th>
<th>Tumour volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.26</td>
<td>1.81</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>13.37</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>14</td>
<td>25.62</td>
<td>2.62</td>
<td>2.1</td>
</tr>
<tr>
<td>21</td>
<td>26.01</td>
<td>2.81</td>
<td>3.8</td>
</tr>
<tr>
<td>28</td>
<td>26.37</td>
<td>3.15</td>
<td>4.5</td>
</tr>
<tr>
<td>35</td>
<td>28.32</td>
<td>3.21</td>
<td>4.8</td>
</tr>
</tbody>
</table>

With tumour growth, there is an increase in cathepsin D concentration compared to the uninoculated rat control group. Thus, one week after inoculation, cathepsin D levels reach an average of 13.37 nM, compared with 11.26 nM in the control group. After another week, the concentration of cathepsin D almost doubles (25.62 nM); then the increase is not so high so that at 5 weeks it will reach 28.32 nM (Figure 2, Table 1).
Sialic acid has the same growth profile in correlation with tumour volume. Serum sialic acid levels were found to increase with tumour development, and almost doubled at 35 days after inoculation (3.21 mM), compared to the time of inoculation (1.81 mM), suggesting appearance of metastases. Two days after inoculation, the sialic acid levels are 2.62 mM, and after another 2 weeks the sialic acid level reaches 3.15 mM (Figure 2, Table 1). Catepsin D and sialic acid (AS) had increasing values during the evolution of the Walker 256 carcinosarcoma, which is consistent with literature data suggesting that these biomarkers play a direct, regulating role in tumour progression and metastasis.

![Figure 3](image3.png)

Figure 3. Correlation analysis between soluble biomarkers of cell proliferation and tumour volume (*p<0.05, **p<0.05, ***p<0.05)

There is a positive correlation between cathepsin D concentration and serum sialic acid level, both parameters having high values during the evolution of the Walker 256 carcinosarcoma. Levels of these serum biomarkers also correlate with tumour dimensions, therefore with tumour growth (Figure 3).

3.5. Evaluation of the oxidative stress in Walker 256-bearing tumours

The Walker 256 inoculated Wistar albino rat is an experimental model close to an "ideal" model to monitor in vivo the oxygen metabolism and the determination of reactive oxygen species in the context of the tumour progression and development of the angiogenesis processes. Therefore, another objective of our study was the identification of the role of reactive oxygen species in the angiogenesis signaling cascade. Thus, after the in vivo experimental model, represented by the induction of Walker 256 carcinosarcoma in Wistar rats, the biochemical parameters of oxidative stress were evaluated in the dynamics of tumour growth.

![Figure 4](image4.png)

Figure 4. Serum levels of ROS in Walker 256 tumour-bearing rats
Similar to cathepsin D or sialic acid parameters, the lipid peroxide values increased from 5.42 μM / 100 mL (before inoculation) to 6.18 μM / 100 mL 7 days post-implant and 8.75 μM / 100 mL 14 days after inoculation. The obtained results indicate an increase in oxidation reactions on lipids, by increasing MDA values over 2 times. Growth is dynamic, proportional to tumour development, and once again suggests that the tumour is the inducer of reactive oxygen species. This is due to the fact that the lipids have a large distribution in the body and in the cell membrane, the first attack of free oxygen radicals being at this level (Figure 4, Table 2).

Total albumin thiols are products of oxidative degradation of sulfur-containing proteins. There is an increase in the determined values at a lower rate than that of the oxidative degradation of the proteins, confirming our data and suggesting that the primary target of the oxidative attack represents the lipids and only then, with the intensification of the chain reactions, when excess free radicals are produced, the protein molecules are also attacked. In addition, they are more protected by their structures.

<table>
<thead>
<tr>
<th>Days from tumour inoculation</th>
<th>PX (μM)</th>
<th>SH (μM)</th>
<th>AO (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>54.21 ± 6.35</td>
<td>311±14</td>
<td>669+53</td>
</tr>
<tr>
<td>7</td>
<td>61.82 ± 2.89</td>
<td>335±11</td>
<td>738+66</td>
</tr>
<tr>
<td>10</td>
<td>72.96 ± 5.93</td>
<td>367±9</td>
<td>887+81</td>
</tr>
<tr>
<td>14</td>
<td>87.5 ± 6.78</td>
<td>375±15</td>
<td>953+46</td>
</tr>
<tr>
<td>21</td>
<td>78.34±13.21</td>
<td>368±32</td>
<td>987+73</td>
</tr>
</tbody>
</table>

Increasing oxidative stress is also seen by increasing levels of thiols in the blood. These are secreted by the tumour and their level is directly correlated with the increase in tumour volume and the number of days from the implant. Thus, one week after inoculation, SH increases from 311 μM to 335 μM, at 14 days reaching 375 μM (Figure 4, Table 2).

![Figure 5](image)

*Figure 5. Correlation analysis between levels of ROS species in Walker 256 tumour-bearing rats (**p<0.05, ***p<0.05, ****p<0.05)*

Once formed, reactive oxygen species are rapidly decomposed by antioxidant systems. As with PX and SH, the most prominent AO increase occurs in the first week of inoculation, from 660 μM to 990 μM (Figure 4, Table 2).

Positive correlation was also found between the ROS parameters detected during the evolution of the Walker 256 carcinosarcoma: SH vs. PX and AO, PX vs. AO (Figure 5).
Conclusions
Growth of transplanted Walker 256 tumours is independent of the age and weight of the animal at inoculation; it is characterized by rapid and uniform growth, rare regression; it is an adaptable tumour, does not not metastasize contralaterally in organs, such as lungs, hurrying the death of the animal during experiment. Walker tumours are well vascularized and have numerous venous dilatations. The success rate of tumour transplantation is over 80%. Walker 256 tumour maintained through subcutaneous grafts in Wistar rats is metastasized lately in loco-regional lymph nodes, and it can be preserved through serial passages from one animal to another.

Catepsin D and sialic acid had increasing values during the evolution of the Walker 256 carcinosarcoma, and a positive correlation was found between cathepsin D concentration and serum sialic acid level during the evolution of the Walker 256 carcinosarcoma. The tumour has a particular behaviour, and by circumventing the mechanisms of growth control, it manages to develop a series of adaptive mechanisms to stress factors. Positive correlations also exist between ROS parameters during the evolution of the Walker 256 carcinosarcoma. In response to the increase in cellular oxidative stress, the tumour produces these sulfur-containing proteins (glutathione, cysteine, thioredoxin, etc.) in excess as an endogenous protection measure. As a result, the increase in tumour protein oxidation is not always received by the body as an oxidative stress factor and does not activate the natural antioxidant protection systems at the same time.

Acknowledgments
We are grateful for the support offered by the POS-CCE O2.2.1. 433/ 2012 project.

References