TOTAL PROTEOLYTIC ACTIVITY AND LEVELS OF THE MAIN PROTEINASE INHIBITORS IN BLOOD PLASMA OF MICE BEARING LEWIS LUNG CARCINOMA UPON DEVELOPMENT OF RESISTANCE TO CISPLATIN

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The aim of the study was to evaluate the total proteolytic activity (TPA) and the content of α₁-proteinase inhibitor (α₁PI) and α₂-macroglobulin (α₂M) in the blood plasma of mice with Lewis lung carcinoma (LLC) upon the development of resistance to cisplatin. Methods: Experimental LLC model with different sensitivity to cisplatin was obtained by sequential subcutaneous transplantation of LLC cells from cisplatin-treated animals. TPA, α₁PI and α₂M levels were evaluated by standard biochemical methods.

Results: It has been shown that the development of LLC resistance to cisplatin is accompanied by the increase of TPA activity and the level of the main proteinase inhibitor — α₁PI. Despite the high level of α₁PI in the resistant variant of LLC compared to parental tumor, the increase of TPA/α₁PI ratio indicated the deficiency of that inhibitor in the blood of mice bearing cisplatin-resistant tumors, that promotes metastasis. The growth of both resistant to cisplatin LLC and sensitive variant was accompanied with the reduction of the α₂M concentration.

Conclusions: Upon the development of resistance to cisplatin in vivo the shift in the balance between proteinases and their inhibitors toward activation of TPA simultaneously with the increased metastasis is taking place.

Key Words: total proteolytic activity, α₁-proteinase inhibitor, α₂-macroglobulin, drug resistance, cisplatin, metastasis, Lewis lung carcinoma.

Resistance to chemotherapy limits the efficacy of treatment of cancer patients [1] and in part is dependent on the expression of specific proteins in tumor cells that “neutralize” chemotherapeutic agents [2–5]. The experiments performed in vitro have shown that the development of resistance to cisplatin may be accompanied by alteration in expression levels of some proteolytic enzymes [6–8], and moreover, proteinases may be involved in blockage of drug-induced apoptosis [9–10].

It is known that proteolytic enzymes, in particular serine proteinases are causally involved in cancer progression [11–13]. Various studies have demonstrated the importance of serine proteinase inhibitors in regulation of the activity of serine proteinases. The serum proteinase inhibitor, α₁PI (α₁-antitrypsin) is a member of the serpin superfamily of serine proteinase inhibitors, that maintain homeostasis through regulation of a number of proteolytic processes. It is known that tumor cells are producing not only proteinases [14, 15], but also the α₁PI [16, 17]. This inhibitor prevents the contact of immunocompetent cells with tumor cells and depresses T-killers [18]. It has been reported that metastasis and recurrence are associated with an increase of α₁PI concentration [19–21]. Another multifunctional inhibitor, α₂M, controls the activity of the wide spectrum of proteolytic enzymes [22].

Presently there is a lot of publications on chemoresistance of tumors, but the role of proteolytic enzymes and their inhibitors in the development of chemoresistance in vivo remains poorly studied yet.

In a previous study [23], the authors investigated TPA and changing of antiproteinases levels in the blood plasma of the rats bearing Guerin carcinoma with the induced resistance to doxorubicin. It was shown that the development of resistance to doxorubicin is accompanied by shifted balance between proteinases and proteinase inhibitors in the blood plasma, in particular, TPA and α₂M content decrease and the increase of α₁PI level.

The aim of the study was to evaluate the changes in the total proteolytic activity (TPA) and the main proteinase inhibitors levels (α₁PI and α₂M) in blood plasma of mice with metastatic Lewis lung carcinoma (LLC) upon the development of resistance to cisplatin.

MATERIALS AND METHODS

In this study, 125 male adult C57B1/6 mice weighting 22–25 g bred in the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine (Kyiv, Ukraine) were used. All animal procedures were carried out according to the rules of Ethic Committee.

LLC transplantation has been made as follow: suspension of tumor cells (1.5 x 10⁶ cells in 0.02 ml of the medium 199) was injected subcutaneously to femoral zone of each animal. Cisplatin resistance of LLC has been induced by sequential cell transplantation from the mice treated previously with cisplatin. Mice have been divided into two groups: control (group 1) and experimental (group 2). At days 7–9 after LLC transplantation mice from the group 2 received cisplatin (EBEWE, Austria) at the doses of 1.2 mg/kg intraperitoneally (5 injection in total with 1 day intervals).

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Abbreviations used: α₂M — α₂-macroglobulin; α₁PI — α₁-proteinase inhibitor; LLC — Lewis lung carcinoma; TPA — total proteolytic activity.
Tumors relapsing after chemotherapy was finished (days 23–25 after LLC transplantation) have been used for further transplantations, and experiment has been repeated by the same schedule. In total, 27 courses of chemotherapy were performed. Then 4 groups of animals have been studied: Group 1 — mice with parental (sensitive) LLC after the first injection; Group 2 — LLC with induced resistance to cisplatin (after 9 courses of chemotherapy), LLC/R<sub>9</sub>; Group 3 — LLC with the induced resistance to cisplatin (after 19 courses of chemotherapy), LLC/R<sub>19</sub>; Group 4 — LLC with induced resistance to cisplatin (after 27 courses of chemotherapy), LLC/R<sub>27</sub>. The content of TPA, alpha1PI and alpha2M were determined in blood plasma of the mice with wild type and resistant tumors after 9, 19 and 27 courses of the chemotherapy. The samples of blood plasma were stabilized by 3.8% of sodium citrate solution (9:1) (Merk, Germany) and centrifuged at 1500 g for 10 min at 4 °C.

The determination of the TPA in the blood plasma was carried out according to the method [24], that allows to determine the trypsin-like protease activity of the blood plasma, and can be used for the determination of the endogenous proteolytic activity of the neutral serine proteinases. The activity of the neutral serine proteinases was expressed in µmol arg/min·ml of blood plasma. The content of alpha1PI in blood plasma (g/l) was determined by its ability to suppress the N-benzoyl-DL-arginine-p-nitroaniline (BAPNA, Sigma) hydrolysis with trypsin. The alpha2M content (g/l) was determined by its ability to form a complex with trypsin insensitive to the soybean inhibitor (Sigma, USA). The studied indexes were determined at the days 10, 15, 20, 25, 28 after the tumor inoculation. Blood plasma indexes of intact animals were used as the reference one. The statistical analysis of the data was performed using Student’s t-test. The differences p < 0.05 were considered as significant.

**RESULTS AND DISCUSSION**

The study of biochemical indexes in the blood plasma of animals have been determined in the dynamics of LLC development: at the day 10th after LLC transplantation (initial stage of tumor growth), 15th (intensive tumor growth), 20th (period of active dissemination), 25th (the appearance of lung metastases), 28th (terminal stage of the tumor growth).

It was found that cisplatin inhibited the parental LLC growth by 46% and decreased the lung metastases number by 58%, in the case of LLC/R<sub>9</sub> — by 26 and 46%, LLC/R<sub>19</sub> — by 15 and 26%, LLC/R<sub>27</sub> — by 3 and 11%, respectively. In total, we have registered the increase of the lung metastases by 44.7% upon development of resistance to cisplatin (LLC/R<sub>27</sub> group versus LLC group).

In Table 1, the results of determination of TPA in the blood plasma of mice with LLC upon the development of resistance to cisplatin are presented. At the 10th day after tumor transplantation, TPA level in the blood plasma of mice with LLC increased compared to the control, but at all other time points it was lower than the respective control values. So, elevation of TPA level was observed at the initial stage of tumor growth, and its reduction — at the stages of active dissemination and metastasis of LLC.

**Table 1.** Total proteolytic activity in blood plasma of LLC-bearing mice compared to intact control (%)

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Days after tumor transplantation</th>
<th>TPA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC</td>
<td>0</td>
<td>100.0± 10.7</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;9&lt;/sub&gt;</td>
<td>100.0± 10.7</td>
<td>116.6± 10.4</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;19&lt;/sub&gt;</td>
<td>100.0± 10.7</td>
<td>100.0± 10.7</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;27&lt;/sub&gt;</td>
<td>100.0± 10.7</td>
<td>116.6± 10.4</td>
</tr>
</tbody>
</table>

Note: *p < 0.05 compared to intact control.

The LLC/R<sub>9</sub> growth was also accompanied by the elevation of TPA level in the blood plasma of animals on 10th and 15th days after LLC transplantation, and its decrease at later terms. It is necessary to note that LLC/R<sub>9</sub> tumors appear with a 4–5 days delay compared to parental LLC.

The development of the LLC/R<sub>19</sub> has been characterized by elevation of TPA content in the blood plasma of mice on 10th day after tumor transplantation; in this group that index tends to increase on the days 15, 20, 25 (however, non-significantly compared to the control). At the same time the development of LLC/R<sub>27</sub> was accompanied by marked TPA elevation at all studied time points.

So, the obtained data have demonstrated that the activity of neutral serine proteinases in the blood plasma of LLC-bearing mice is gradually elevating upon the development of tumor’s resistance to cisplatin, especially in the periods of intense tumor growth and metastasis. Upon intensive growth of LLC/R<sub>9</sub> TPA level decreased, whilst that of LLC and LLC/R<sub>19</sub> is equal to the control values, and LLC/R<sub>27</sub> — was significantly elevated.

The determination of the content of main protease inhibitor in blood plasma of LLC-bearing mice upon the development of the resistance to cisplatin (Table 2) have shown the next peculiarities: on days 10, 15, 20 after the tumor transplantation alpha1PI level has been significantly higher in blood plasma of mice with LLC compared to the control (p < 0.05), in LLC/R<sub>9</sub> that index was also elevated compared to the control, in LLC/R<sub>19</sub> — was increased at all stages of the measurements of growth excluding the 10th day. The development of LLC/R<sub>27</sub> has been characterized by increased level of alpha1PI except 28th day.

**Table 2.** Alpha1PI content in blood plasma of LLC-bearing mice compared to intact control (%)

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Days after tumor transplantation</th>
<th>Alpha1PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC</td>
<td>0</td>
<td>16.6± 10.8*</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;9&lt;/sub&gt;</td>
<td>16.6± 10.8*</td>
<td>16.6± 10.8*</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;19&lt;/sub&gt;</td>
<td>16.6± 10.8*</td>
<td>16.6± 10.8*</td>
</tr>
<tr>
<td>LLC/R&lt;sub&gt;27&lt;/sub&gt;</td>
<td>16.6± 10.8*</td>
<td>16.6± 10.8*</td>
</tr>
</tbody>
</table>

Note: *p < 0.05 compared to intact control.
Thus, the blood plasma of all tumor-bearing animals is characterized by significantly increased level of alpha1PI compared to that in blood plasma of intact animals.

The determination of the alpha2M level in blood plasma of experimental animals has shown that this index was significantly decreased in blood plasma of all LLC-bearing mice upon development of the cisplatin-resistance compared with intact control (Table 3), however, no significant differences in alpha2M content were found between the studied groups of LLC-bearing animals with different degree of cisplatin resistance.

Table 3. Alpha2M content (g/l) in blood plasma of LLC-bearing mice compared to intact control

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Days after tumor transplantation</th>
<th>Alpha2M (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>LLC</td>
<td>1.2 ± 0.1</td>
<td>1.14 ± 0.85</td>
</tr>
<tr>
<td>LLC/R9</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>LLC/R9m</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>LLC/R9t</td>
<td>1.2 ± 0.92</td>
<td>1.02 ± 0.82</td>
</tr>
<tr>
<td>LLC/R9o</td>
<td>0.06</td>
<td>0.07*</td>
</tr>
<tr>
<td>LLC/R9r</td>
<td>1.2 ± 0.83</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Note: *p < 0.05 compared to intact control

So, the presented results have shown that the development of resistance of LLC to cisplatin is accompanied by the imbalance between proteolytic and antiproteolytic activities shifted to the activation of proteinases. This process occurs despite marked elevation of alpha1PI level in blood serum of LLC-bearing mice compared to control group and is reflected in the value of TPA/alpha1PI ratio. TPA/alpha1PI is nearly equal during period of active metastasis of LLC and LLC/R9 (Table 4) that points on the decreased proteolytic potential via elevated content of alpha1PI, which is responsible for 90% antitrypsin activity in blood plasma [24]. TPA/alpha1PI ratio is significantly higher at LLC/R9 during period of active metastasis compared with this value in LLC and LLC/R9o groups. The highest TPA/alpha1PI and simultaneously the highest metastasis has been registered in LLC/R9r-bearing animals. One may conclude that the increase of TPA/alpha1PI value in blood plasma reflects the aggressiveness of the tumor.

Table 4. TPA/alpha1PI ratio in LLC-bearing mice with different resistance to cisplatin

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Days after tumor transplantation</th>
<th>TPA/alpha1PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>LLC</td>
<td>1</td>
<td>0.99</td>
</tr>
<tr>
<td>LLC/R9</td>
<td>1</td>
<td>0.96</td>
</tr>
<tr>
<td>LLC/R9m</td>
<td>1</td>
<td>1.57</td>
</tr>
<tr>
<td>LLC/R9t</td>
<td>1</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Another important observation is that the development of cisplatin-resistance was accompanied by the decrease of alpha2M content in blood serum of tumor-bearing animals. Decreased level of alpha2M could be considered as an additional factor promoting tumor growth, since alpha2M has cytotoxic effect toward cancer cells [25].

In conclusion, the progression of the parental LLC is accompanied by the decrease of TPA in blood plasma of experimental animals, whilst the development of cisplatin resistance is characterized by gradual TPA increase. Thus, we have shown that the balance of proteinase-inhibitor system in the blood plasma of LLC-bearing mice is shifted toward activation of proteolytic enzymes and deficiency of the alpha1PI upon the development of cisplatin-resistance. According to reported data [26–28], the development of cisplatin resistance in vitro is associated with an increase of the expression level of serine proteinases. The present study has demonstrated such association in vivo.

REFERENCES