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Quantitative Regulation of Melanoma Growth in the Host by Tumor-Specific Serpins in Blood Serum is a Main Reason for Inefficient Tumor Treatment

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1. Introduction

1.1 Estimation of efficiency of experimental chemotherapy

The analysis of the phenomena of experimental oncology should begin with an estimation of efficiency of chemotherapy (Goldin A et.al. 1983). All criteria allow the distinction into two groups. Either or not an increase of life span (ILS) of tumor-bearing animals can be reached after treatment. Cytostatic drugs destroy a part of the tumor and, as a result these mice live longer than mice without treatment. The conclusion from this observation is that it is necessary to kill as much tumor cells as possible. Another possibility is that cytostatic drugs lead to inhibition of tumor growth, but an increase of life span does not occur. This observation shows that life span of tumor-bearing animals does not only depend on the number of killed tumor cells. To elucidate the mechanism of this unexpected phenomenon, we performed the following experiment. C57Bl mice were transplanted with $6.1x10^6$ of melanoma B16 cells into the right back hip. The animals were divided into three groups. The control group was left untreated. The second group received doxorubicin i.p. (7 mg/ kg of body weight) 24 hours after melanoma cell transplantation. Another group received doxorubicin i.p. (7 mg/ kg of body weight) 9 days after transplantation. Each group contained 10 mice. In figure 1 the average life spans of animals is shown. In the control group, the average life span was 41.7 ± 1.8 days. The average life span in the second group was 67.6 ± 1.5 days ($p<0.05$ vs control). The ILS of mice after treatment by doxorubicin was 62 %. The average life span of mice in the third group was 40.8 + 2.5 days ($p>0.5$ vs control).
An inhibition of a tumor growth observed between day 12 and 19 was 100% (Fig. 2). (taken from own data).
It can be concluded that the best ILS tumor-bearing mice could be reached by treatment of animals with doxorubicin injected 24 hours after tumor transplantation. This result led us to the hypothesis that tumor cells have no time to establish in the host during the first 24 hours after transplantation. At this time, dependence between the number of killed tumor cells by

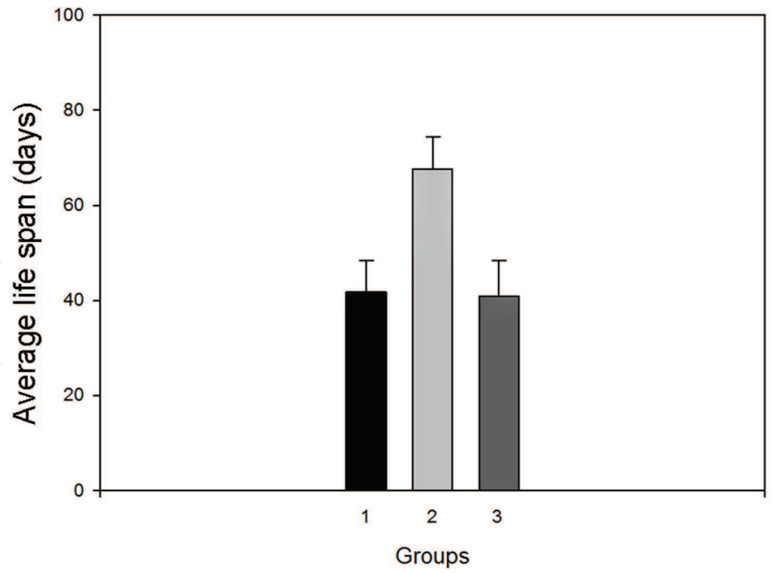

Fig. 1. Influence of the time interval between tumor transplantation and treatment with doxorubicin on average life span of B16 melanoma-bearing mice. 1. The control group was not treated with doxorubicin. 2. In the second group of animals which doxorubicin was injected 24 hours after tumor transplantation. 3. In the third group of animals, doxorubicin was injected 9 days after transplantation

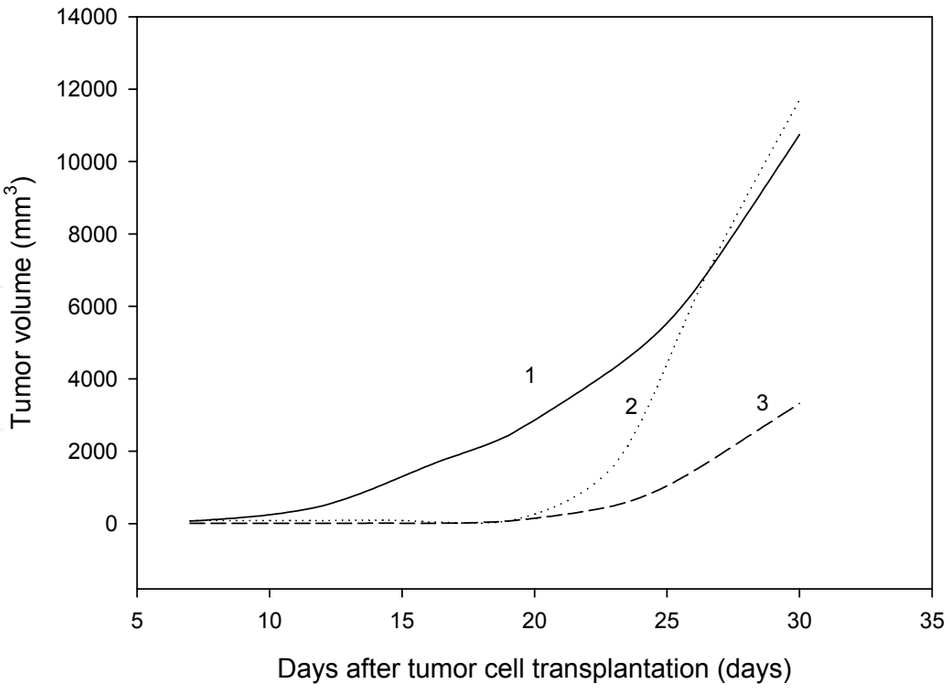

Fig. 2. Influence of the time interval between tumor transplantation and treatment by doxorubicin on tumor volume of B16 melanoma-bearing mice. Details see Figure 1. (taken from own data)

chemotherapy and ILS of animals was observed. Nine days after transplantation, tumor cells were integrated into the host. Then a molecular growth program for the tumor was set on. This program of tumor growth determines the life span of tumor-bearing animals. Therefore, a dependence between the number of killed tumor cells by chemotherapy and ILS of animals is not observed in advanced tumors.

1.2 Acceleration of tumor growth after removal of primary tumor

It is a well-known phenomenon that metastatic growth is boosted after removal of the primary tumor. We addressed the question, whether growth of metastases in mice with removed primary tumor really leads to a decrease of average life span. Therefore, we performed the following experiment. Mice with implanted B16 melanoma were used in the experiment. The conditions of tumor implantation are described above. All animals were divided into three groups. The first group contained control animals. In the second group, the tumor was removed 24 hours after injection of tumor cells. In the third group, the tumor was removed 9 days after implantation of the tumor. In figure 3, the results of the influence of removal of the primary tumor on average life span of mice are shown. The average life span of mice in the control group was 39.3 ± 2.4 days, in mice which received treatment 24 hours after tumor inoculation more than 70 days ($p<0.05$ vs control), and in mice where the tumors were removed 9 days later 38.3 ± 2.9 days ($p<0.5$ vs control). In the last group, in animals metastatic growth in lungs was observed. 1. After transplantation of tumor cells they were integrated into the host. 2. As a result of this integration, a program of tumor growth was activated in the host. 3. This program restored eliminated cells so that the average life span of animals did not change.

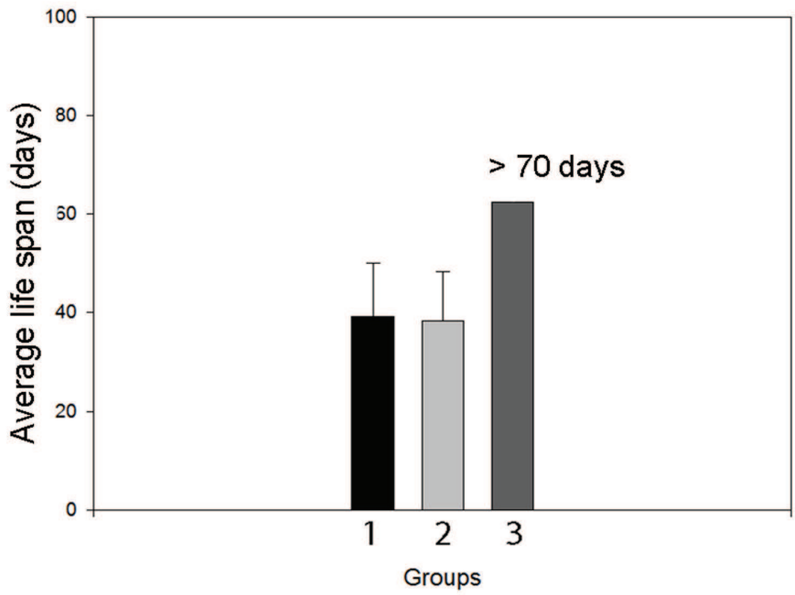

Fig. 3. Influence of the time interval passed between tumor transplantation and its removal on average life span of B16 melanoma-bearing mice. 1. Control group without tumor removal. 2. Group of animals where primary tumors were removed 9 days after transplantation of B16 melanoma cells. 3. Group of animals where primary tumors were removed 24 hours transplantation of B16 melanoma cells (taken from own data)

In the literature, this phenomenon is known as acceleration of tumor growth after removal of primary tumor. In contrast, our results did not show an acceleration of tumor growth. Animals with removed tumor died as rapid as control mice. After removal of the primary tumor the growth characteristics changed, and the tumor actively metastasized. From our point of view it is, therefore, correct to speak about a phenomenon of stability of tumor growth (Donenko et al., 1995).

1.3 Specific stimulation of metastatic growth

It is very easy to prove that this program of tumor growth in the host is tumor specific (Sitdikova et al., 2007). We performed the following experiment: C57Bl/6 mice or hybrids of first generation (F1) were used. B16 mouse melanoma cells were transplanted to one flank, and Ehrlich carcinoma cells were transplanted into the other side of the mice. B16 melanoma cells are black colored and it metastasizes after removal of primary tumor into the lung. Ehrlich carcinoma cells have a white color and metastasized after removal of primary tumor into the belly lymph nodes. When the melanoma was removed, melanoma-derived black metastases occured in the lung, while white metastases were not found in the belly lymph nodes. Removal of Ehrlich carcinoma was followed by the development of carcinoma-derived white metastases in the belly lymph nodes, but not of black metastases in the lung. Since melanoma and carcinoma cells were transplatend to the same mice, the constitution of growth factors, e.g. vascular endothelial growth factor (VEGF) etc. was identical. However, the response of B16 melanoma and Ehrlich carcinoma was specific. Therefore, we conclude that the mechanism which stimulated the restoration of tumor cells is specific.

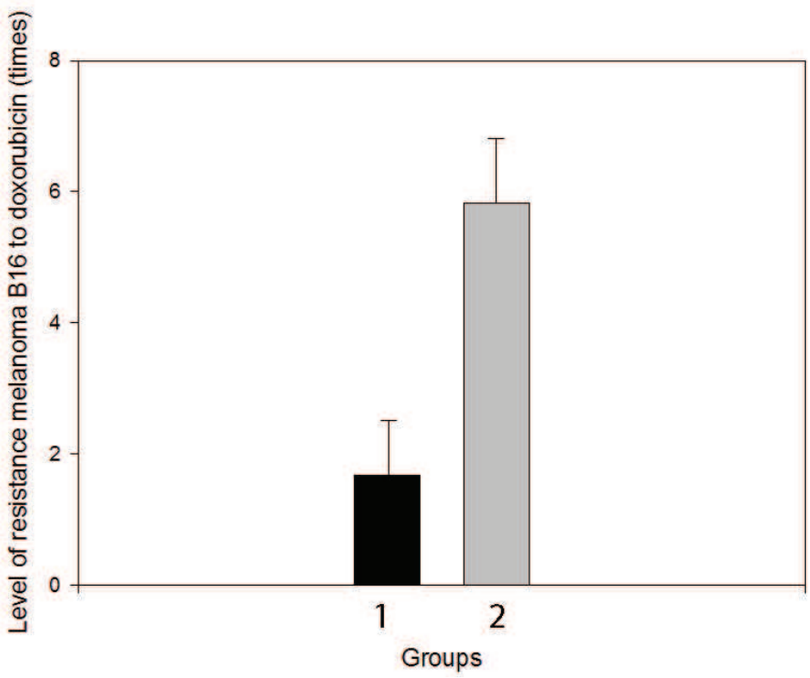

Fig. 4. Level of resistance of B16 melanoma cells towards doxorubicin after six tumor passages. 1. Doxorubicin was injected 24 hours after tumor transplantation. 2. Doxorubicin was injected 9 days after B16 melanoma transplantation to mice (taken from own data)

1.4 Induction of drug resistance by a tumor-specific mechanism

In another experiment, B16 melanoma-bearing C57Bl/6 mice received doxorubicin (5 mg/kg body weight). All animals were divided into two groups. In the first group, doxorubicin was injected 24 hours after tumor transplantation. After the tumors reached a volume of 1000 mm^3, the melanoma cells were transplanted to healthy mice. These mice received doxorubicin also 24 hours after tumor cell transplantation. Afterwards, the entire cycle was repeated. Animals of the second group were treated with doxorubicin 9 days after tumor cell inoculation. After the tumors reached a volume of 1000 mm^3, the melanoma cells were transplanted to healthy mice. These mice were injected with doxorubicin also 9 days after tumor transplantation. Subsequently, this cycle was repeated. The entire experiment was stopped, when the mice in the second group a reduction of tumor volume was not observed anymore after doxorubicin treatment. We concluded that resistance of melanoma cells to doxorubicin has developed (Donenko et al., 1991). The degree of resistance of B16 melanoma cells towards doxorubicin after six tumor passages was 1.7 (±0.5), if the drug was injected 24 hours after tumor transplantation. However, a significantly higher resistance (5.9 (±1.4)-fold, $p<0.05$) was observed after six passages, if doxorubicin was injected 9 days after tumor transplantation (Fig. 4).

We conclude that the mechanism restoring number of tumor cells after doxorubicin chemotherapy accelerated the selection of drug-resistant B16 melanoma cells (Sukhanov et al., 1991).

1.5 Influence of blood serum proteins of tumor-bearing mice on the growth rate of primary tumors

Experiment

Melanoma cells (10^5 cells) were injected into the right back leg of C57bl/6 mice. Then animals were divided into three groups. The first group of animals was the control group. The second group of animals received i.p. 0.4 ml of serum blood of healthy C57Bl/6 within one hour after tumor transplantation. The third group of animals were injected with 0.4 ml of B16 tumor-bearing of serum blood within one hour after tumor transplantation As shown in figure 5, acceleration of tumor growth was only observed in animals which received serum from tumor-bearing animals, but not in animals receiving serum of healthy animals or in control mice. Thus, we conclude that blood serum of tumor-bearing mice contains tumor-specific factors which accelerate tumor growth (Donenko et al., 1995; Donenko et al., 1997; Sitdikova et al., 2003).

1.6 Concomitant immunity after removal of primary tumors

This phenomenon has been described as resistance of animal to a second challenge tumor transplantation of the same tumor (Gorelik, 1983). It was found that this phenomenon is not restricted to tumors, but can also be observed with infective and parasitic diseases. This phenomenon is dependent on quantitative parameters of infections, parasitic diseases and tumor growth (Gorelik, 1983).

We performed the following experiment: Animals were divided into four groups. The first group consisted of healthy animals. Then, C57bl/6 mice were injected with three groups of B16 melanoma cells into the right back leg. Each mouse was inoculated with 10^6 B16 melanoma cells. The second group of animals was the control group. Then, 9 days later 10^5 melanoma cells were subcutaneously transplanted to two groups (group number 3 and 4) of

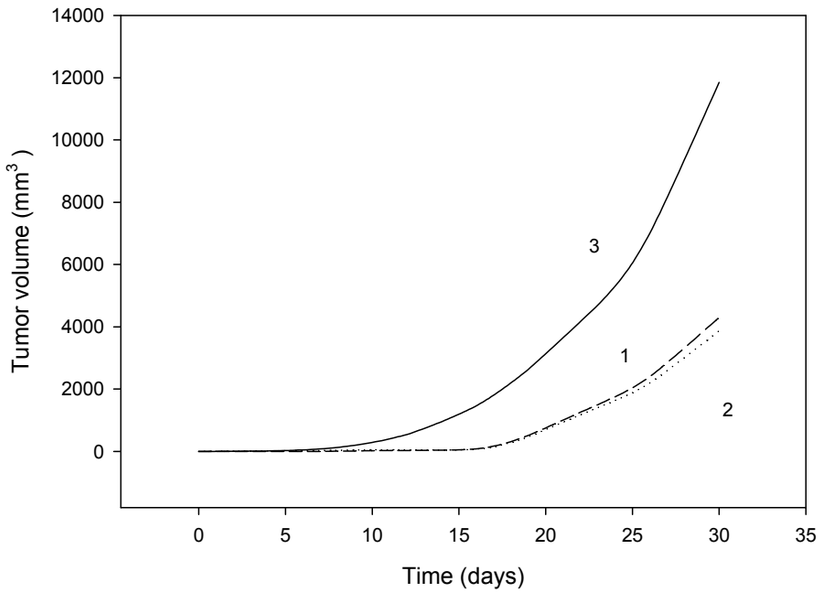

Fig. 5. Influence of serum blood proteins of tumor-bearing mice on growth of primary B16 melanoma. 1. Control group. Serum blood proteins were not injected into mice. 2. Group of mice which received serum blood proteins of healthy mice. 3. Group of mice which received serum blood proteins of B16 melanoma-bearing mice (taken from own data)

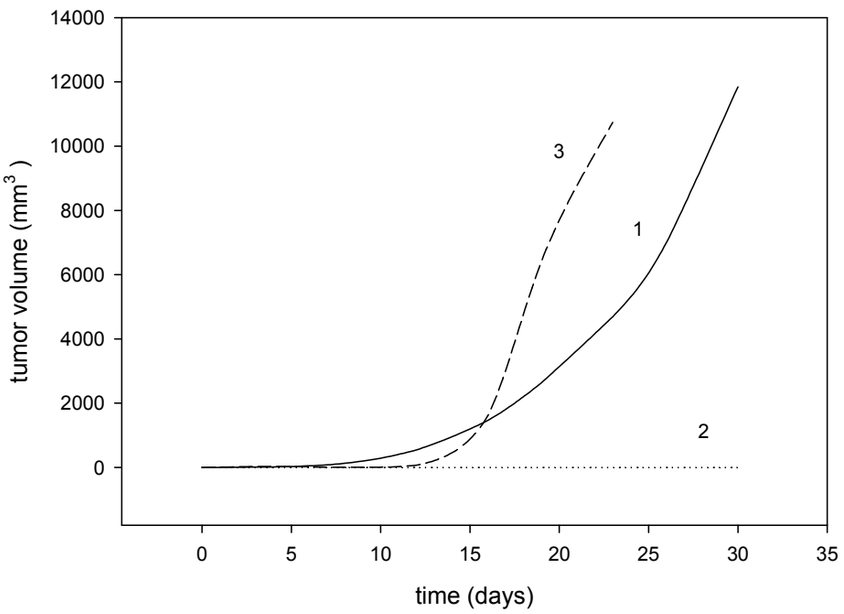

Fig. 6. Influence of concomitant immunity on growth of a second challenge with B16 melanoma transplant and subcutaneousB16 melanoma metastases after removal of the primary tumor. 1. Growth of a second challenge B16 melanoma transplant in healthy mice (group 1). 2. Growth of a second challenge in B16 melanoma-bearing mice with primary tumor or with removed primary tumor (groups 3 and 4). 3. Growth of subcutaneous metastases in mice with removed primary tumor (group 3) (taken from own data)

tumor-bearing mice and the group of healthy animals(second challenge tumor transplantat). At the same day, primary tumors were removed in mice of one of the two groups (group number 3). Figure 6 shows the results of a second challenge of tumor transplantation is shown. In the tumor-bearing animals of the third and fourth groups, the second challenges did not result in tumor growth (line 2). In the healthy animals of the first group, tumor growth was observed after a second challenge(line 1). For removal of primary tumors, the mice of the fourth group were fixed by means of clips. At the location, where the clips were fixed, we observed the growth of subcutaneous B16 melanoma metastases. Growth of these metastases is shown in line 3 of figure 6 (group 3). The growth of these metastases was faster in comparison with the growth of the second challenge. Furthermore, the growth of these metastases was associated with a damage of the hypodermic layers by the clips. It is an unexpected, but remarkable result that both the naturally occurring lung metastases and the artificial, clip-induced subcutaneous metastases were observed (group 3).
We conclude that tumor cells grow at strictly defined locations and with strictly defines speed. This phenomenon is associated with a still unknown mechanism. This mechanism determines location and growth rate of melanoma cells (Donenko et al., 1992).

2. Identification tumor-specific factors in blood serum

Previously, it was hypothesized that a growth-promoting factor is released by the primary tumor (Weiss, 1952). Attempts to identify this factor were unsuccessful during the past 50 years. The phenomenon of accelerated metastatic tumor growth after removal of the primary tumor represents a major reason for the relapse of the disease (Peeters et al., 2006). The mechanisms how the removal of the primary tumor influences metastatic growth are still not understood. By using six different tumor models, Fisher et al. hypothesized the existence of a growth-stimulating factor in the blood serum of animals after removal of the primary tumor (Fisher et al., 1989).
Fisher at al. hypothesized that this growth factor is present in an inactive form and becomes active over time. An important fact for this hypothesis is that the maximal acceleration of metastatic growth was observed only within the first 24 h after removal of the primary tumor, a time period which is too short to affect neo-angiogenesis of tumors or to restore volume of the removed tumor. Rather than hypothesizing the release of a growth-promoting factor by the primary tumor as done by Fisher and co-workers, we assume that a growth-stimulating factor is constantly produced by the tumor-bearing host (see: Phenomenon of influence of proteins of serum blood of tumor-bearing mice on growth rate of primary tumor.). If this assumption is true, the removal of the primary tumor by surgery would then lead to an increase of its concentration in blood serum and to acceleration of metastatic growth. However, increasing concentrations of this factor in the serum activate a negative feedback loop resulting in a subsequent decrease of this factor in the serum and a retardation of metastatic growth. Hence, decreased proliferation of tumor cells within 24 h after removal of primary tumor might indicate the existence of a negative feedback loop. A balance between growth stimulation and retardation leads to a net increase metastatic growth (Donenko et al., 2009).
To prove our assumption, we have chosen Ehrlich ascitic carcinoma as test model And then to repeat the same effect on melanoma Cloudman S91. Ehrlich ascitic carcinoma cells have been removed together with ascitic fluid by means of a syringe. During this procedure, only the number of tumor cells in the tumor-bearing host changed. Other factors possibly influencing the biological effects of our procedure were excluded in this experimental

setting, i.e. anesthetics, surgical trauma, bleeding, change of concentration of tumor cell in the ascitic fluid, growth inhibition of the tumor by contact interaction, and the influence of tumor angiogenesis. All these factors might have stirred Fisher and co-workers investigating this phenomenon. Hence, the hypothesis is that a tumor-stimulating factor is produced by the tumor-bearing host itself. Only its continuing production by the host might accelerate the growth of the remaining tumor cells.

2.1 Tumor-specific factors in blood serum. How can they influence growth of a tumor?

The cell cycle analysis of ascitic fluids by flow cytometry was carried out seven days after inoculation of tumor cells. The initial percentage of proliferating cells in ascitic fluids was about 15 %. The removal of ascitic liquids caused an increase of proliferating cells to 30.8 % after 24h. This result is comparable with data of Fisher et al. After 48h, the percentage of proliferating cells decreased again to 16% and remained constant after 120h (17%) (Figure 7) (Donenko et al., 2009).

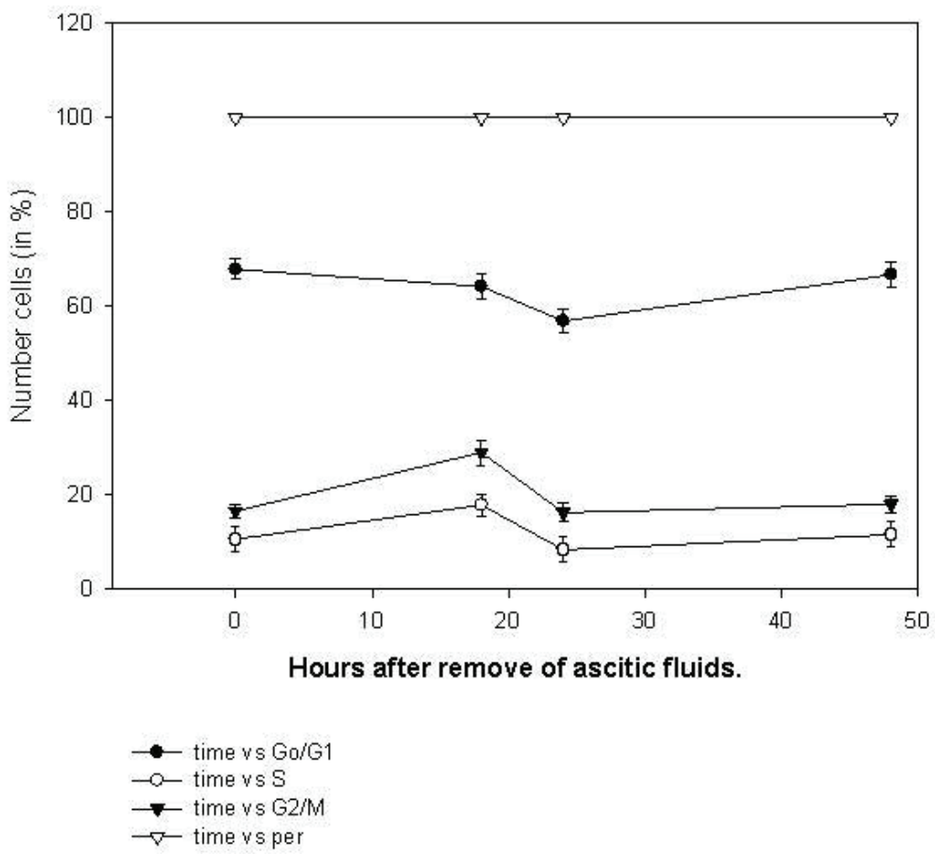

Fig. 7. Influence of removal ascitic fluids on distribution of Ehrlich carcinoma cells and peritoneal cells on cell cycle. —●— - Influence of removal ascitic fluids on distribution of Ehrlich carcinoma cells in the G0/G1 cell cycle phase. —○— - Influence of removal ascitic fluids on distribution of Ehrlich carcinoma cells in the S cell cycle phase. —▼— - Influence of ascitic fluid removal in distribution of Ehrlich carcinoma cells in the G2/M cell cycle phase. —▽— - Influence of ascitic fluid removal on distribution of peritoneal cells in G0/G1 cell cycle phase. X-axis, time (hours after remove ascitic fluids); Y-axis, number of cells (in %)

Representative histograms and gel electrophoreses of three independent experiments are shown (taken from Donenko et al., 2009 with permission).
By means of flow cytometry, it is possible to distinguish Ehrlich carcinoma cells from normal peritoneal cells. Peritoneal cells were completely in the G0/G1 phase (100%) (Figure 7). 1. Serum tumor-specific factor can increase or decrease tumor cells mitotic activity. A tumor-specific factor of the serum blood determines the mitotic index of tumor cells. It means that a serum tumor-specific factor is necessary for the entry of tumor cells into mitosis. Without such a serum tumor-specific factor the division of tumor cells is impossible. Phenomena like this have been previously described (Fisher B, et. al., 1989). For all these phenomena, an increase of life span after treatment of advanced B16 melanoma B16 was not observed. Hence, the phenomenon of stabile tumor growth can possibly explained by the regulation of mitotic tumor cell activity by a serum tumor-specific factor.

2.2 Negative feedback loop

It is important to note that our published experiments one the resistance of mice towards tumor transplantation have been successfully repeated by other scientists.
Decreased proliferation of tumor cells within 24h after removal of primary tumor might indicate the existence of a negative feedback loop. We hypothesized that removal of a primary tumor activates a growth-inhibitory process towards the remaining tumor cells in the tumor-bearing host. To prove this possibility, peripheral blood leukocytes (PBLC), spleen leukocytes (SLC), and PC were transferred from animals with removed ascites tumor into healthy animals together with Ehrlich tumor cells. A scheme of the experimental setting is shown in Figure 8.

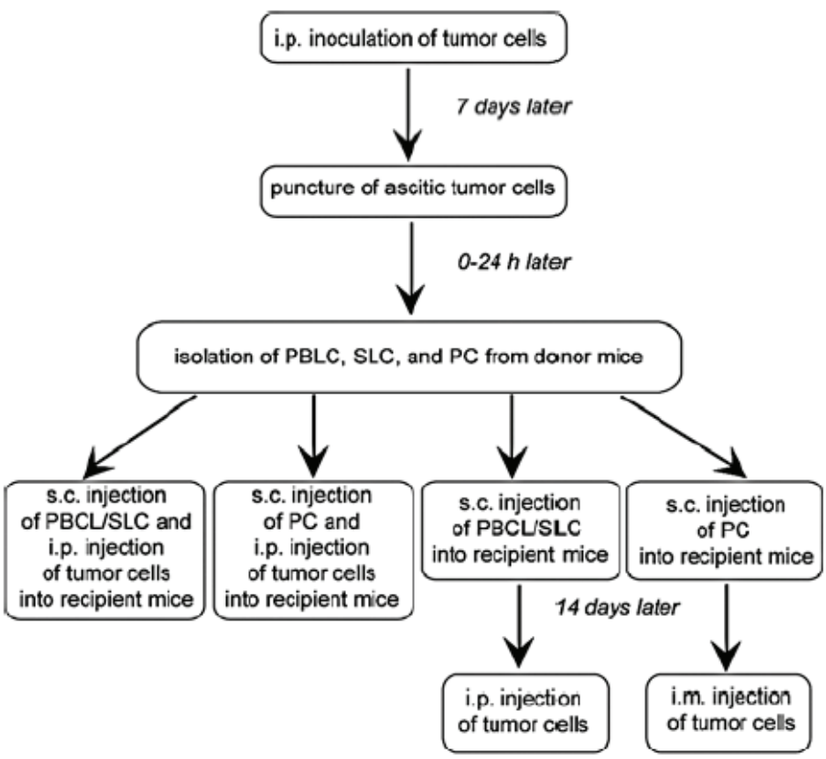

Fig. 8. Diagram of the experimental setting

No. group	Time after tumor removal (hours)	Cell type	Resistance of mice (%)
1.	0	SLC	0
2.	0	PBLC	0
3.	0	PC	0
4.	1	SLC	0
5.	1	PBLC	0
6.	1	PC	0
7.	4	Cells of spleen	0
8.	4	SLC	0
9.	4	PBLC	0
10.	5	PC	15.6 ± 2.8
11.	5	PBLC	0
12.	5	PC	12.5 ± 5.0
13	6	SLC	32.5 ± 5.0
14.	6	PBLC	35.0 ± 5.7
15.	6	PC	42.5 ± 3.1
16.	8	SLC	47.5 ± 8.3
17.	8	PBLC	27.5 ± 4.3
18.	8	PC	28.1 ± 4.5
19.	10	SLC	22.5 ± 5.0
20.	10	PBLC	0
21.	10	PC	0
22.	12	SLC	0
23.	12	PBLC	0
24.	12	PC	0
25.	24	SLC	0
26.	24	PBLC	0
27.	24	PC	0
28.	48	SLC	0
29.	48	PBLC	0
30.	48	PC	0
31.	120	SLC	0
32.	120	PBLC	0
33.	120	PC	0

Table 1. Time kinetics of donor cells to inhibit Ehrlich carcinoma cells in recipient mice after removal of Ehrlich carcinoma in donor mice. Tumor cells (1×10^6 cells) were injected into mice together with peripheral blood leukocytes (PBLC), spleen leukocytes (SLC), or peritoneal cells (PC) (3×10^6 cells). Ehrlich carcinoma cells were i.m. injected, if PC were co-applied. Ehrlich carcinoma cells were i.p. transplanted, if PBLC or SLC were co-applied (taken from Donenko et al., 2009 with permission)

No. group	Time after tumor removal (hours)	Origin of cells	Resistance of mice (%)
1.	0	SLC	0
2.	0	PBLC	0
3.	0	PC	0
4.	1	SLC	0
5.	1	PBLC	0
6.	1	PC	0
7.	4	SLC	0
8.	4	PBLC	0
9.	4	PC	0
10.	5	SLC	8.1 ± 4.5
11.	5	PBLC	0
12.	5	PC	86.7 ± 5.8
13	6	SLC	93.1 ± 5.7
14.	6	PBLC	66.7 ± 5.8
15.	6	PC	87.5 ± 5.0
16.	8	SLC	98.5 ± 3.8
17.	8	PBLC	58.0 ± 16.4
18.	8	PC	82.5 ± 14.9
19.	10	SLC	6.7 ± 5.8
20.	10	PBLC	0
21.	10	PC	0
22.	12	SLC	0
23.	12	PBLC	0
24.	12	PC	0
25.	24	SLC	0
26.	24	PBLC	0
27.	24	PC	0
28.	48	SLC	0
29.	48	PBLC	0
30.	48	PC	0
31.	120	SLC	0
32.	120	PBLC	0
33.	120	PC	0

Table 2. Time kinetics of donor cells to induce resistance towards Ehrlich carcinoma in untreated, healthy recipient mice after removal of Ehrlich carcinoma in donor mice. Peripheral blood leukocytes (PBLC), spleen leukocytes (SLC), or peritoneal cells (PC) (3×10^6 cells) were subcutaneously injected. Ehrlich carcinoma cells were i.m. injected, if PC were co-applied. Ehrlich carcinoma cells were i.p. transplanted, if PBLC or SLC were co-applied. Then, 14 days after donor cell injection 1×10^6 carcinoma cells were applied (taken from Donenko et al., 2009 with permission)

PBLC, SLC, PC were taken at different time points after ascitic tumor removal (0 – 120 h). This procedure suppressed tumor growth in 20-40% of the recipient animals, if PBLC, SLC, or PC were taken only 6-8 h after tumor removal in the donor animals (Table 1). A follow-up of those mice, which showed resistance towards tumor development after injection of PBLC, SLC, or PC revealed that these mice maintained tumor resistance for at least four months, even if the number of transplanted tumor cells increased from 10^3 to 40×10^6 cells. This follow-up experiment is still continuing to explore the long-term effect of this phenomenon.
To address the question, whether resistance towards tumor development is induced by the host or by the tumor cells, which where co-transplanted along with PBLC, SLC, or PC, we performed another set of experiments. PBLC, SLC, or PC were injected into mice, and Ehrlich tumor cells were inoculated 14 days later (see the scheme in Figure 8) (taken from Donenko et al., 2009 with permission).
The results in Table 2 demonstrated that tumor resistance occurred in the same time frame (6-8 h) and was more frequent than in the previous experiment (60-80% in Table 2 versus 20-40% in Table 1). This may be taken as a clue that the tumor-suppressive activity mediated by PBLC, SLC, or PC was induced by the host. Possibly, 6-8 h represents a necessary time frame for recovery of the animals from surgical interventions and also activation of defense phenomena as observed in our set of experiments.
We observed this phenomenon not only in Ehrlich carcinoma, but also in Cloudman S91 melanoma (Table 3). Recipient mice injected with PBCL, SLC, or PC from operated donor animals revealed less frequently tumors, if these cells have been taken 6-8 h after operation. The percentage of mice resistant towards tumor development could be increased, if these cells were injected repeatedly. Single injection of these cells resulted in a percentage of 20% mice with resistance towards melanoma development. Double transplantation increased the number of resistant mice up to 40-70 %, and after triple transplantation resistance was found in up to 100% of mice. These results strongly speak for tumor-suppressive effects conferred by the host organism.

2.3 Possible activation mechanism for the negative feedback loop

In Figure 9, the influence of removal of ascitic fluid on binding of FITC-labeled serum glycoproteins is shown in a time-dependent manner. Initially (0 h) the percentage of positive cells is zero or very low. One hour after removal of ascitic liquid the percentage of positive cells increased up to 6%, and after 7h up to 21%. A maximum of about 40% was reached 24 h after removal of the ascitic liquid. These data show that the changes in PBLC, SLC, and PC as well as in serum glycoprotein immunoreactivity occurred after 6-8 h and 18 h, respectively (Donenko et al., 2009). The chemical nature of this factor is unknown. We favor another explanation, which points to a factor initially produced by the host itself. This factor is absorbed by the tumor and is necessary for cell proliferation. After removal of the tumor, the concentration of this factor drastically increases. Then, the increased concentration of this factor in blood serum leads to a negative feedback loop resulting in a subsequent decrease of this factor in the serum. This poses the question, as to why such a hypothetical negative feedback loop is activated. A possible answer to this question is provided by our flow cytometry data. Using forward and sideward scattering, we observed a change in size of tumor cells after partial removal of the ascitic fluid. This change in tumor cell size might be associated with a change in the interaction of these cells with molecules in the ascitic fluid. This assumption is important to postulate a negative feedback loop. Ascitic fluids and blood contain many polar and hydrophobic molecules. These chemical groups are located

on the surface of cells and biomacromolecules. A concentration change of these chemical groups might lead to the dissociation of these molecules from the surface or to the formation of molecule complexes. Complexes might be formed between biomacromolecules, between biomacromolecules and cells, and as conglomerates of cells. A formation of cell conglomerates may be dangerous for an organism, as it causes the development of the so-called "sludge" syndrome. For example, this syndrome occurs after dehydration of an organism or after sepsis. Dehydration causes an increase of the erythrocyte number in blood. As a result, erythrocytes form monetary column-formed structures (Hinshaw, 1996; Moore et al., 2003).

No. group	Time after tumor removal (hours)	Cell type	Number injection of donor cells	Resistance of mice (%)
1.	6	SLC	Single	27.5 ± 4.9
2.	6	PBLC	Single	12.5 ± 5.0
3.	6	PC	Single	17.5 ± 5.0
4.	6 7	SLC + PBLC	Double	40 ± 8.2
5.	6 7	PC + SLC	Double	66.7 ± 5.8
6.	6 7 8	PBLC + PC + SLC	Triple	86.7 ± 5.7
7.	6	PC	Triple	98.5 ± 3.8
8.	6	PBLC	Triple	82.5 ± 5.0
9	6	SLC	Triple	98.5 ± 3.8

Table 3. Influence of modes of application of donor cells on the growth of Cloudman S91 melanoma in mice. Peripheral blood leukocytes (PBLC), spleen leukocytes (SLC), or peritoneal cells (PC) (3×10^6 cells) were subcutaneously injected. Single, double, or triple injections were performed. Fourteen days after donor cell injection, 1×10^6 Cloudman melanoma cells were subcutaneously applied (taken from Donenko et al., 2009 with permission)

Therefore, we studied the interaction of blood serum glycoproteins with peritoneal cells of mice. Our results indicate an increased binding of glycoproteins with peritoneal cells after removal of ascitic liquid. A formation of conglomerates of the remaining tumor cells in the ascitic fluid was observed after visual inspection. Hence, it is worth speculating that partial removal of ascitic fluid causes a condition in the remaining cells similar to the"sludge" syndrome. Such a condition might be dangerous to the tumor-bearing host and might possibly lead to a switch to compensatory mechanisms. As a result, the affinity of biomacromolecules to tumor and peritoneal cells might be changed. Affinity changes of proteins to cells can be measured as alterations in their glycosylation pattern. Indeed, we have shown that glycosylation of blood serum proteins changed within 6h after removal of ascitic fluid. It is reasonable to assume that lymphocytes can carry out protein glycosylation reactions by recognition and dissociation of formed complexes, which can then be released into biological fluids such as ascitic fluid (Sitdikova et al., 2005).

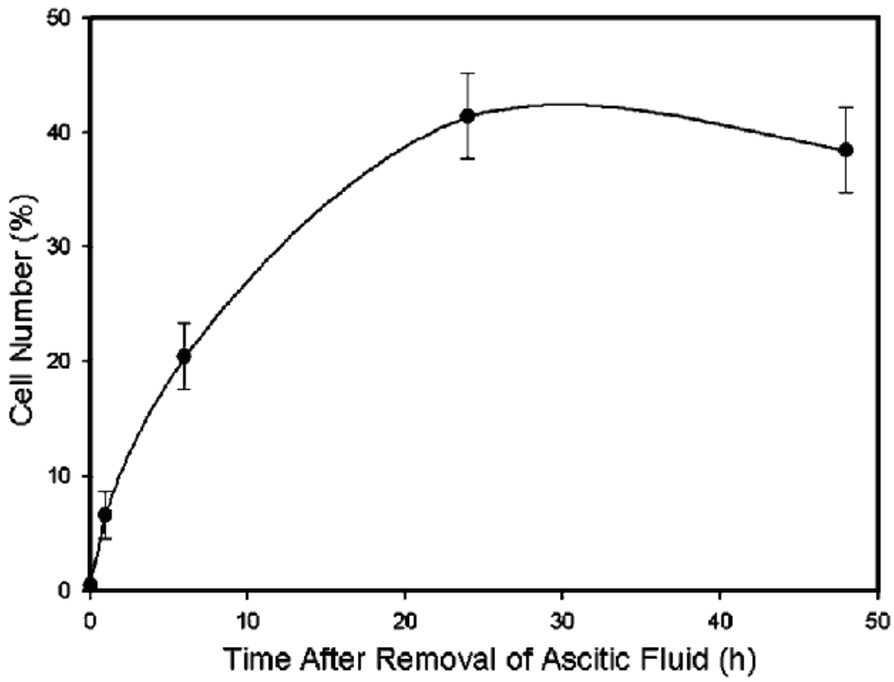

Fig. 9. Quantitative analysis of the time-dependent influence of removal of ascitic fluid on the binding of FITC-labeled serum glycoprotein's from ascitic Ehrlich carcinoma cell-bearing mice after removal of ascitic fluid injected with peritoneal cells received from i.m. transplanted Ehrlich carcinoma-bearing mice (taken from Donenko et al., 2009 with permission)

2.4 Identification of tumor-specific serum factors

We have received very interesting experimental model of development resistance of mice to the transplanted tumor. We have shown that increasing the levels of a hitherto uncharacterized factor induced a negative feedback mechanism, which inhibited the development of this factor by the host. This in turn led to inhibition of tumor growth as shown for Ehrlich carcinoma and Cloudman S-91 melanoma. This can be taken as a clue for the existence of an equilibrium between serum factors and target cells, which might influence each other. The infringement of such a balance resulted in the occurrence of new cellular properties, leading to resistance of mice towards tumor growth. These tumor-suppressing features became apparent upon transfer of blood serum from tumor-bearing mice to healthy mice inoculated with cancer cells. The aim of the next step of investigation was to identify tumor-specific serum factors of mice bearing Ehrlich carcinoma, which have the potential to confer resistance towards tumor development. For this purpose, we incubated isolated immune cells (peritoneal cells or spleen lymphocytes) from intact mice in vitro with blood serum or ascitic fluid from tumor-bearing mice. From our point of view during incubation immune cells from intact mice in serum of blood or in ascitic liquid of tumor-bearing animals in vitro system observes the same infringement of tumor-specific balance which is observed in vivo system in animals with the removed primary tumor. Immune cells which are received from intact mice should to react against a tumor-specific of balance of serum blood proteins or ascitic liquid. Time through which will develop this reaction it is known to us. It makes 7 hours. The experimental design is shown in Figure 10.

The results of the experiments are presented in Table 4. Mice inoculated with PC previously incubated for 7 h with ascitic fluid did not develop tumors at a frequency of 9 from 10 mice (inoculation of Ehrlich carcinoma cells after two weeks) or 100% (inoculation of Ehrlich carcinoma cells three months later). Inhibition of tumor growth was observed in mice injected both with PCs and SLCs 6 - 8 h after infringement of balance between the tumor and the tumor-bearing host. In the next step, the infringement of balance between tumor and host has been simulated in vitro by incubation of PCs and SLCs from mice without tumor with ascitic liquid or its fractions from tumor-bearing mice.

Table 4 shows the data of animals injected with PCs. However, SLCs possessed the same ability to induce resistance mice to tumor growth. Accordingly, it has previously been shown that tumor growth was not inhibited in mice which received PCs and SLCs 4 h after tumor removal. Therefore, the control group of animals received PCs 4 h after incubation with PCs and ascitic liquid or its fractions. This indicates that mice developed resistance towards tumor development. In resistant mice, after i.p. injection of 1×10^6 tumor cells per mice tumor growth was not detected at least for one year. By contrast, resistance towards tumor formation was not observed in the control group and life span of mice was not more than 20 days. To further analyze this phenomenon, we prepared different fractions of the ascitic fluid: fractions with proteins of >300 kDa (fraction 1), of 100-300 kDa (fraction 2), of 50-100 kDa (fraction 3), and of <50 kDa (fraction 4). Furthermore, two glycoprotein fractions were prepared, one with lower affinity than concavalin A, which were eluted by saccharose (fraction 5) and another with higher affinity than concavalin A, which was eluated by methylmannopyranoside (fraction 6). Corresponding controls were prepared for all six fractions. As shown in Table 4, fractions 1 and 2 weakly prevented tumor formation in mice. Only three out of 10 mice were resistance to tumor growth. This resistance towards tumor growth was short. At a repeated injection of ascitic cells three months later resulted in tumor growth in three hour of three all mice. The tumor growth rate in these mice was much less than in the control group. In control group, mice lived less than 20 days compared to two months in the experimental group. A strong prevention of tumor formation was obtained for fractions 3 and 5. Nine out of ten mice which received fraction 3 and seven from ten mice which received fraction 5 were resistant to tumor growth upon tumor cell inoculation two weeks after PCs. This resistance towards tumor growth was much longer. Repeated injections of ascitic cells three months later resulted in inhibited tumor growth in nine out of nine mice which received fractions 3 and six out of seven mice which received fraction 5. The best protection rate was measured using fraction 3. Resistance towards tumor formation was found in nine out of ten mice (two-week regimen) and nine out of nine mice (three-month regimen). Fractions 4 and 6 as well as all control fractions did not confer resistance on mice towards tumor development.

As a next step, we investigated the active fractions 1, 2, 3, and 5 by LC/MS, in order to identify their molecular constituents. 40 proteins were identified in the activated protein fractions from tumor-bearing mice. To determine, which of these differentially regulated serum proteins were functionally linked to resistance to tumor development, we incubated protein fraction 5 with PCs for 7 h, removed PCs by centrifugation, and subjected the fraction to LC/MS-MS. The comparison of the protein fraction before and after incubation showed that serpin (α-1-antitrypsin) was absent from the PC-incubated fraction, indicating that this protein was bound to PCs and, thereby, purged from the protein fraction. This result obtained by LC/MS-MS was confirmed by gel electrophoresis and MALDI-TOF analyses. And at least one protein has appeared after 7 hours incubation PC-incubated

fraction. As determined by mass spectrometry, this band represents cathepsin L1, with 93.9% intensity coverage (Donenko et al., 2010).

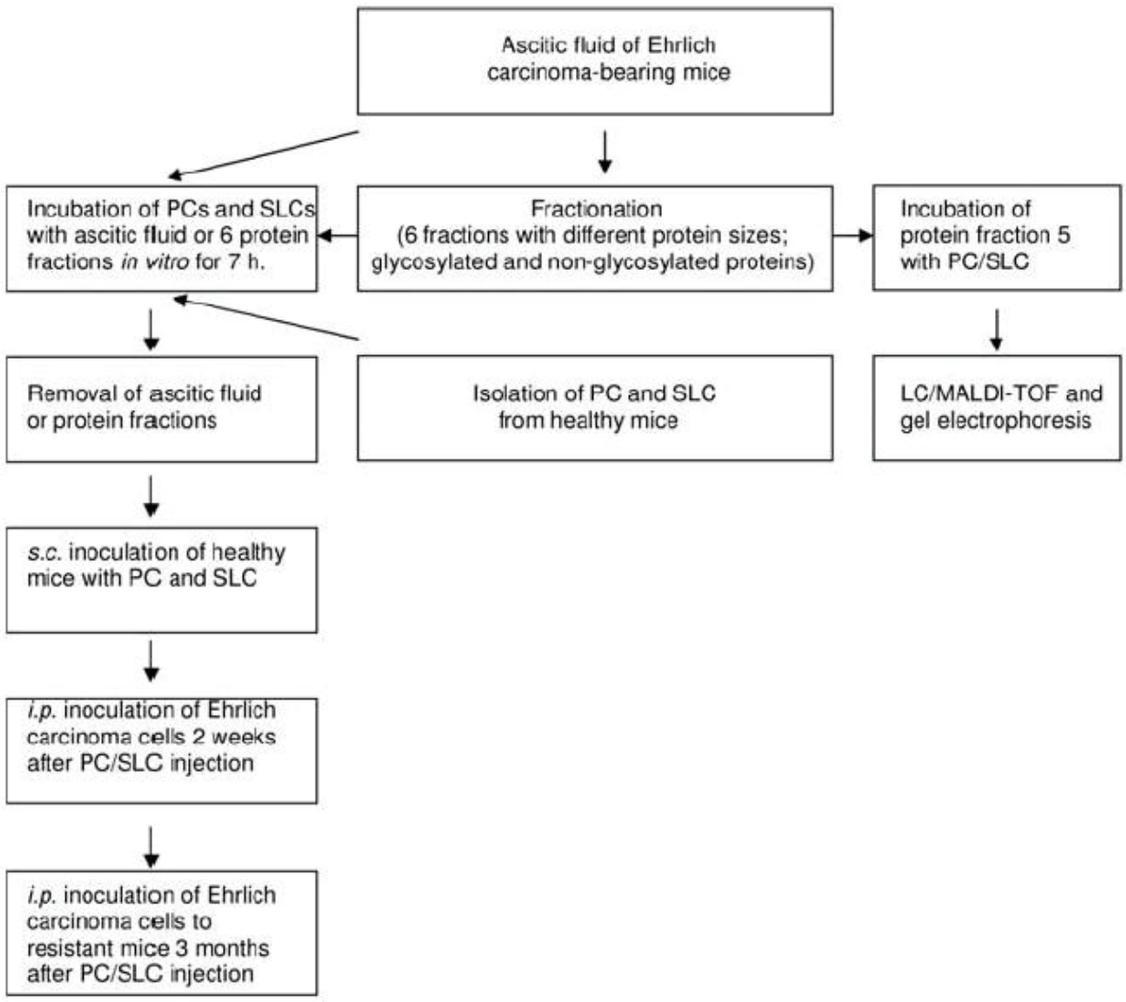

Fig. 10. Diagram of the experimental setting (taken from Donenko et al., 2010 with permission)

Peritoneal cells (PCs) (3×10^6 cells) were treated in vitro with protein fractions from ascites of tumor-bearing mice and subcutaneously injected into healthy mice. Fourteen days after donor cell injection, 1×10^6 Ehrlich carcinoma cells were i. p. applied. Then, three months later mice which were resistant to tumor development after the first tumor cell injection (14 days) received another injection of the same type of tumor cells (3×10^6 cells).

The proteomic analyses finally led to the identification of serpin (α-1-antitrypsin) as candidate protein to explain the biological activity of fraction 5. We identified 40 different proteins, but only one protein of this fraction, serpin, disappeared in our experimental setting. Serpin is a major protein of blood serum with an amount of 200-400 mg in 100 ml blood serum. This indicates an eminent role of this protein in biological processes. The full exhaustion of serpin was accompanied by loss of tumor-specific activity of fraction 5. Absorption of serpin from fraction 5 by PC correlated with secretion of cathepsin L1.

Interestingly, it has previously been reported that squamous cell carcinoma antigen (SSCA), another serpin member inhibited cathepsin L (Takeda et al., 1995, Kato H, 1996.). It can, therefore, be speculated that the purging of serpin (α-1-antitrypsin) in our investigation might lead to a re-expression of cathepsin L1, which is otherwise repressed in the presence of serpin (α-1-antitrypsin).

N	Fraction of ascitic fluid	Number of resistant mice compared to all mice 14 days after donor cells injection#	Number of resistant mice compared to all mice three months after donor cells injection
1.	Ascitic fluids	9/10	9/9
	Control	0/10	0/10
2.	Fraction 1: fraction of ascitic fluid with proteins of more than 300 kDa	3/10	0/3#
	Control	0/10	0/10
3.	Fraction 2: fraction of ascitic fluid with proteins in a range of 100 to 300 kDa	3/10	0/3#
	Control	0/10	0/10
4.	Fraction 3: fraction of ascitic fluid with proteins in a range of 50 to 100 kDa	9/10	9/9
	Control	0/10	0/10
5.	Fraction 4: fraction of ascitic fluid with proteins of less than 50 kDa	0/10	
	Control	0/10	
6.	Fraction 5: first fraction of ascitic fluid with glycoproteins in a range of 50 to 100 kDa	7/10	6#/7
	Control	0/10	0/10
7.	Fraction 6: second fraction of ascitic fluid with glycoproteins in a range of 50 to 100 kDa	0/10	
	Control	0/10	

Table 4. Influence of different protein fractions of ascitic fluid from tumor-bearing mice on peritoneal cells and spleen lymphocytes from healthy mice on induction of resistance towards Ehrlich carcinoma growth in healthy recipient mice. # Mice still alive after the first injection of ascitic cells were injected with tumor cells again three months later. Repeated tumor cell injection caused tumor growth. The tumors growth in early resistant mice was very slowly (about 30 – 60 days) and without development of ascitic fluid. In control groups, mice developed ascitic fluid and died within 20 days. (taken from Donenko et al., 2010 with permission).

2.5 (Patho)physiological processes of quantitative organ (tissue) regulation in the body

Not only tumor tissues, but all tissues of an organism may participate in the complex balance of biomacromolecules, an assumption which is supported by the literature. Removal

of a kidney, a part of the liver, a salivary gland or a plaintive gland causes an increase in the proliferative activity of the remaining organ or gland (Urie et al., 2007 ; Haxhija et al., 2007; Martin et al., 2008; Juno et al., 2002; Nelson et al., 2002; Vlastos et al., 2008; Aussilhou et al., 2008; Wilms et al., 2008). A factor appears in blood serum which enhances cell proliferation as early as 12 h after small bowel resection (Juno et al., 2003.). There are several indications in the literature that a serum factor causes an increase in vascular endothelial growth factor (VEGF) after organ resection (Parvadia et al., 2007; Schrijvers et al., 2005). This factor is not VEGF, since VEGF has no organ or tumor specificity. When mice are transplanted with two tumors of different colors, such as melanoma (black) and carcinoma (white), removal of the carcinoma is followed by the development of carcinoma-derived white metastases in the belly lymph nodes. When the melanoma is removed, melanoma derived black metastases occur in the lung. Hence, the proposed serum factor underlying this metastasis route may be revealed to have organ and tumor specificity (Sitdikova et al., 2007). The occurrence of lipomatosis after cosmetic surgery also supports our view of complex balances (Ginat et al., 2008; Puttarajappa et al., 2008; Jowett et al., 2008; Pandzic Jaksic V et al., 2008). Goshtasby et al described a case of isolated symmetrical lipomatosis of soft tissue overlying the trochanters that developed a recurrence after liposuction treatment (Goshtasby et al., 2006). Removal causes responses in the organism, such as increased cell proliferation in the remaining tissue. These data further strengthen the assumption that a balance exists between the biomacromolecules of tissues and glands from one side and the biomacromolecules of serum blood proteins from another side.

Another line of evidence comes from studies by Scheiffarth et al. (1967) and Warnatz et al. (1967). The authors showed that the spleen cells of a donor with chemically-induced hepatitis caused hepatitis in the recipient. The ability of donor lymphocytes to keep and transmit disease-mediating information about an illness is difficult to explain. However, taking our hypothesis into account, there are interesting parallels between both phenomena. Lymphocytes contribute to a healthy balance in the organism. In the above mentioned studies, lymphocytes were taken from an organism suffering from chemically-induced hepatitis. Therefore, donor lymphocytes from an unbalanced organism can transmit this information to the recipient organism, who subsequently also develops a diseased state. In the our present study, donor cells (PBLCs, SLCs or PCs) were obtained from mice with extracted tumors. We assumed that these mice had developed a condition inhibiting tumor growth, and therefore sought to identify the occurrence of a similar condition in the recipient mice. We found that the injection of PBLCs, SLCs or PCs from donor mice collected at a maximum of 6-8 h after tumor removal protected the recipient mice from tumor growth. Furthermore, this tumor-stimulating factor might be specific for each individual tumor. This specificity might be achieved by individual glycosylation patterns of blood serum.

Bearing in mind that inhibitors of proteases are frequently of low molecular weight (e.g. the pancreatic inhibitor of trypsin has a weight of only 6 kDa (Stryer L., 1981), it is noteworthy that serpin is an inhibitor of elastase in neutrophils and has a molecular weight of 45 kDa. Furthermore, serpin is a thermosensitive and glycosylated protein. It can be speculated that this protein may perform complex cellular functions, rather than non-specific enzyme inactivation. Concerning lability of protein activity, it has been reported that during isolation of serpin only 0.22% of the total serum protein fraction retained specific enzymatic activity (Mistry et al., 1991.). Serpins play a central role in the regulation of a wide variety of (patho)physiological processes, including coagulation, fibrinolysis, inflammation, development, tumor invasion, and apoptosis (Kummer et al., 2004; Law et al., 2006.).

Furthermore, serpins may protect parasites against the immune systems of the host (Yan et al., 2005). Taken together, it can be hypothesized that serpins represent a tissue- and tumor-specific anti-proteinase. The specificity of anti-proteinase activity is frequently determined by glycosylation (Lonberg-Holm et al., 1987; Silverman et al., 2004). Different glycosylation patterns of serpins might exert specific protection function in fatty tissue, muscular tissue, skin or other organs and tissues.

Thus, growth, development, and quantity of a tissue in an organism might not only be determined by growth factors, but also by a balance of protases and anti-proteases. Such proteases are produced by immune cells located in corresponding tissue or organs, while anti-proteases are produced in the liver. The fact that immune cells generate tissue-specific proteinases supports this hypothesis (Miller et al., 2002.). The phenomenon of regulation of different metabolic processes by specific proteases and antiproteases is well-known, e.g. for coagulation, fibrinolysis, kallikrein/kinin/kininogen system), but we hypothesize that this principle is even more global. The fact that serpins are ancient and well-conserved proteins throughout evolution may be taken as a clue for an ancient immune system, which controls the structure of organs and tissues. The question for the existence of such an ancient immune system arises. Previously, we have described the activity of a protease of Klebsiella pneumoniae (Trishin et al., 2004.). Surprisingly, this protease did not destroy secreted proteins from Klebsiella pneumoniae itself, whereas mammalian target proteins were effectively destroyed. This clearly indicates that microbial proteases can recognize and distinguish own and foreign proteins. This capability may be interpreted as an early step in the evolution of immunity. The quantitative regulation of a tissue in a host by serpins is determined by the suicide properties of this molecule, i.e. one molecule of serpin inactivates one molecule of proteinase in a stoichiometric manner. The production of cathepsin L1 in parallel to serpin activity allows us to assume that this proteinase may destroy other cells which are not subject to protection in this tissue or organ (homing effect and concomitant immunity phenomenon). The production of cathepsin L1 by PCs may contribute to Ehrlich carcinoma growth in mice. Possibly, cathepsins can cause protein receptors to be shed from a surface of tumor cells. As a result of removal (shedding) of these protein structures from the tumor cell, it cannot be fixed and grow in this tissue. Quantitative regulatory processes are characteristic not only for a tumor, but also for chronic infections and for parasites (Gorelik E, 1983). This explains the phenomenon of homing, where mislocated cells in a tissue or organ cannot exert their function or are destroyed. Growth factors do not possess such specificity. Examples illustrating this phenomenon are growth factors of blood vessels. Their activity increases after removal of healthy tissue, e.g. liver tissue, or a tumor. However, accelerated growth of blood vessels is observed only in the healthy removed tissue or in the removed tumor. Vessel sprouting in other tissues with other tissue-specific proteinases does not take place.

3. Hypothesis: Regulation of cellular physiological and patho-physiological homeostasis by proteinase-antiproteinase balance

The modern theory of immunity cannot explain many phenomena. One of the basic statements of immunity is that the immune system recognizes and distinguishes own from own antigens in another place. However, one striking example which is in contrast to this basic rule of immunity is “homing” effect, when own cells from one organ are stopped in another organ, although there are cells from the same orgamism. It is known that such «own» cells of an organism cannot further develop if thes are dislocated in the body. This

phenomenon has been termed "homing" effect. The "homing" effect is the phenomenon when cells of a organism can develop only in the organ or tissue where they have been generated. We speculate that concomitant immunity is a special case of this "homing" effect. Concomitant immunity is the phenomenon, when "foreign" cells (either tumors, or microbial cells or even parasites) can develop only at that location, where they are fixed in an organism for the first time. Theviological relevance of this phenomenon is enormous. It is assumed that immunity develops by proteases-antiproteases balances at the place of penetration of fungi or other microbial parasites after 9-10 days. Then, the organism develops resistance to all subsequent attacks by these infective agents and prevent infections also at other locations of the host. A similar situation may exist in the case of tumor development. Such chronic conditions of disease (even helminthic invasion) have been described by Gorelik (1983). We point out that immunity does not necessity have to destroy all aliens in the body. If the infective agent does not lead to fatal consequences at the first attack, it may survive at the site of entry into the body. It may not distributed all over the body. This phenomenon has been termed microchimerism and describes the long-term existence of infective microorganisms in an organism (Kallenbach et al., 2011; Miech R.P., 2010). It is possible to explain this phenomenon by our hypothesis. The immune system of an organism does not try to destroy all foreign invaders (being either infective mnicroorganisms or cancer cells), but only recognize them at their site of initial location (homing effect).

The liver synthesizes different serpins with a molecular weight in the range from 45 up to 725 kDa. These serpins represent tissue-, tumor-, or microbial-specific anti-proteinases. The amount of specific serpins determines the quantity of the body`s own tissue or other foreign antigensin this organism. The amount tumor-specific serpins also determines the mitotic index and growth rate of a tumor in a host. For example, we showed that injection of hemoglobin containing complex of blood serum proteins isolated from tumor-bearing animals led to tumor regression. Some regimen of injection of these blood serum proteins decreased the growth rate of Ehrlich carcinoma for about three months. In the control group, the tumor-bearing mice did not live longer than 20 days. Slowly growing tumor cells were transplanted to healthy mice. These mice did not live longer than 20 days. Slowly growing tumor cells had mitotic indeces of 4- 5 % in comparison to 14-16 % in control mice. Blood serum proteins did not reveal cytotoxic activity in vitro. This example shows that the growth rate of a tumor was rather determined by host factors than by specific properties of tumor cells (Donenko et al., 2008).

The homing effect is provided by immune cells, which are associated with the location of infection or tumor development (organ-associated lymphocytes). As described above, these cells have a specific set proteinases in each organ (location) and synthesize specific cathepsins in response to absorption-specific serpins. All these proteinases only attack accessible surface receptors on the cell surface in this organ. Shedding of these receptors stops cellular metabolism and the mitotic cycle cannot be entered. Cells with the intact surface receptors can enter the mitotic cycle in this organ and constitute this organ.

This hypothesis postulates specific tumor-specific serum factors, which allow tumor cells to divide more quickly or more slowly. This hypothesis explains:

1. The mechanism of tumor relapse after drug treatment.
2. The phenomenon of concomitant immunity.
3. The increase of the mitotic index of tumor cells after treatment.
4. The quantitative and specific restoration of a tumor after treatment.
5. The quantitative and specific restoration of normal tissues in the host after treatment.

We envision that this hypothesis opens a new direction of treatment of oncological diseases.

4. Conclusion

For more than 30 years, immense efforts have been undertaken to improve cancer therapy. Still, the cure from their disease is not a common everyday reality for cancer patients. Hakomori described a unusual case of regression of a stomach cancer in a 66 years old women (Hakimori, 1986). After tumor regression, she lived further 22 years without any sign of disease. Hakomori allocated the following distinctive sign of the patient: her blood contained antibodies against all blood groups except her own. The tumor had foreign antigen determinants for the host. The patient experienced a hemotransfusion of 25 ml donor blood. The patient's reaction towards the hemotransfusion was very rough (hemolysis) (Hakomori, 1986). Researchers have established a foreign antigen on the surface of tumor cells and have generated an anticancer vaccine. However, these results could not be repeated. Subsequent vaccines on the basis of tumor-associated antigens were not that effective (Eggermont et al., 2009; Sitdikova et al., 2009).

However, this case shares many common signs with the phenomenon of cure of Ehrlich carcinoma-bearing mice described by us. Ehrlich carcinoma also has antigens foreign to the host. The treatment effect has been obtained after injection of hemolytic products into mice. On the other hand, vaccines generated on the basis of tumor-associated antigens also were not very effective. We assume that in both cases the cure effect was associated with the presence of hemolytic products and a tumor-specific infringement of balance of biomacromolecules. This infringed balance disturbed the synthesis of a tumor-specific factor of blood serum in the host. The absence such a tumor-specific serum factor in the tumor-bearing host led to full tumor regression. The experimental setting described here was repeated by other scientists. Remarkably, a similar tumor regression was also observed in their hands.

In summary, we conlude that it is necessary to block the synthesis tumor-specific factors (serpins) in the tumor-bearing host for a successful treatment of oncological diseases.

The first article about tumor-specific serum factors has been published by us only three years ago. Currently, we are generating mice resistant to the growth of B16 and Cloudman S91 melanoma by manipulating serum proteins. The potential of serpins for the improvement of cancer therapy merits further investigations in the future.

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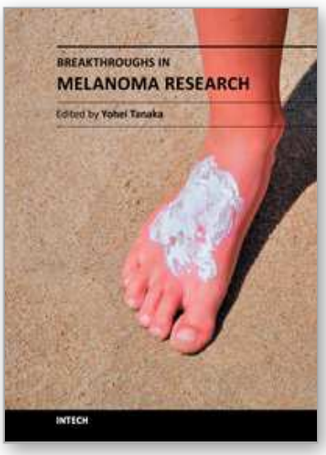

Breakthroughs in Melanoma Research

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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