SPANDIDOS PUBLICATIONS

Induction of resistance to murine tumor development is associated with alterations in the glycosylation of blood serum proteins

F.V. DONENKO[1], R.K. ZIGANSHIN[2], S.M. SITDIKOVA[1], B.S. AMANDZHOLOV[1], M.V. KISELEVSKII[1] and T. EFFERTH[3]

[1]N.N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences; [2]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia; [3]German Cancer Research Center, Pharmaceutical Biology, Heidelberg, Germany

Received December 8, 2008; Accepted March 2, 2009

DOI: 10.3892/mmr_00000126

Abstract. The phenomenon of accelerated metastatic tumor growth following the removal of the primary tumor is a major reason for cancer relapse, caused by underlying mechanisms that are not as yet understood. We hypothesized that a growth-stimulating factor is produced by the tumor-bearing host. This assumption was confirmed by an experiment involving the removal of a primary tumor (ascitic and solid Ehrlich carcinoma cells) from C57B1/6 mice, after which accelerated proliferation was observed in the remaining tumor cells. Peripheral blood leukocytes (PBLCs), spleen leukocytes (SLCs) and peritoneal cells (PCs) were transferred from donor animals with their tumors removed to healthy animals, along with tumor cells. This procedure suppressed tumor growth in 20-40% of the recipient animals when PBLCs, SLCs or PCs were collected 6-8 h after the removal of the tumor. In a second experiment, PBLCs, SLCs or PCs were injected into the mice, and the tumor cells were inoculated 14 days later. Resistance to tumor development occurred within the same time frame (6-8 h) but was more pronounced (60-80%) than in the previous experiment. Ehrlich carcinoma cells affected the binding of FITC-labeled blood serum glycoproteins in a time-dependent manner. Mass spectrometry revealed that the spectrum of glycopeptides in blood serum taken from control mice differed from the spectra of glycopeptides obtained from mice 8-24 h after the removal of Ehrlich carcinoma cells. Comparable effects were also observed with Cloudman S91 melanoma. In conclusion, the inhibition of tumor growth mediated by donor cells (PBLCs, SLCs and PCs) transferred from operated donor animals to recipient animals indicates the existence of a tumor-regulating factor in blood serum. This phenomenon is associated with characteristic alterations in the glycosylation of blood serum proteins.

Correspondence to: Dr Fedor V. Donenko, N.N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, 24 Kashirskoe Shosse, Moscow 115478, Russia
E-mail: fedor.donenko@gmail.com

Key words: anti-tumor activity, cancer therapy, growth-stimulating factor, metastasis

Introduction

The phenomenon of accelerated metastatic tumor growth following the removal of the primary tumor is a major reason for cancer relapse (1). The mechanisms by which the primary tumor influences the growth of remote metastases are not as yet understood. Using six different tumor models, Fisher *et al* (2) hypothesized the existence of a growth-stimulating factor in the blood serum of animals after the removal of the primary tumor. Metastasized tumor cells and micrometastases spread in the body and respond to this growth-stimulating factor, exhibiting accelerated proliferation (3,4). However, this growth-stimulating factor does not influence tumor growth by stimulating tumor angiogenesis (1), as its maximal activity can be observed within 24 h – too short a time period for the neo-angiogenesis of tumors (1,3-6).

Fisher *et al* hypothesized that this growth factor is present in an inactive form and becomes active over time (2). An important finding in support of this hypothesis is that maximal acceleration of metastatic growth was only observed within the first 24 h following the removal of the primary tumor. Rather than hypothesizing the release of a growth-promoting factor by the primary tumor, as proposed by Fisher and co-workers, we assumed that a growth-stimulating factor is constantly produced by the tumor-bearing host. If this assumption is true, then the surgical removal of the primary tumor should lead to an increase in the concentration of growth-stimulating factor in blood serum and to an acceleration of metastatic growth. However, the presence of such increasing concentrations of growth-stimulating factor in serum could activate a negative feedback loop, resulting in a subsequent decrease of the factor in serum and a retardation of metastatic growth. Hence, decreased proliferation of tumor cells within 24 h of the removal of a primary tumor might indicate the existence of a negative feedback loop. A balance between growth stimulation and retardation leads to a net increase in metastatic growth. Such a growth-stimulating factor has yet to be identified. We conducted the present investigation using an experimental model in an effort to do so.

To confirm our hypothesis, we chose Ehrlich ascitic carcinoma as a test model. Previously, we showed that changes in the balance of serum proteins in blood from Ehrlich tumor-bearing mice led to the apoptosis of tumor cells and cured the animals of their tumors (5). Ehrlich ascitic carcinoma cells

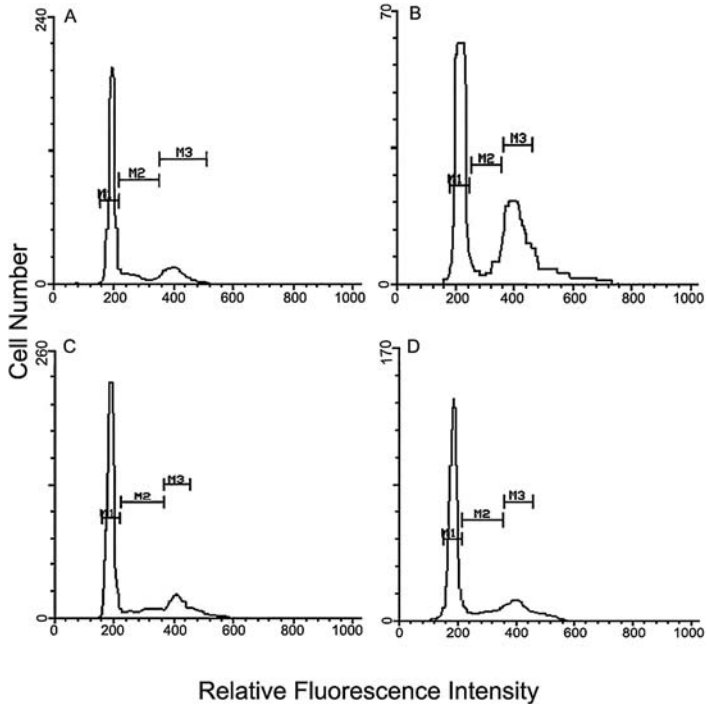

Figure 1. Cell cycle analysis of ascitic Ehrlich carcinoma cells by means of flow cytometry and propidium iodide staining. DNA histograms of ascitic Ehrlich carcinoma cells from mice 0 (A), 18 (B), 24 (C) and 48 h (D) after the removal of ascitic fluid. Representative histograms of three independent experiments are shown.

were 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 the procedure were excluded in this experimental setting, i.e., anesthetics, surgical trauma, bleeding, changes in the concentration of tumor cells in the ascitic fluid, growth inhibition of the tumor by contact interaction, and the influence of tumor angiogenesis. All these factors were of interest in the investigation of this phenomenon conducted by Fisher and co-workers (2). Hence, our hypothesis was that a tumor-stimulating factor is produced by the tumor-bearing host itself. Only its continued production by the host accelerates the growth of the remaining tumor cells. Furthermore, this tumor-stimulating factor is specific to each individual tumor, a specificity that is achieved by individual glycosylation patterns in blood serum.

Therefore, the aim of the present study was to explore the mechanism of inhibition of Ehrlich carcinoma proliferation. We found a negative feedback loop of the regulation of tumor growth 24 h after the removal of ascitic fluid. The time kinetics of this feedback mechanism and its activation by peritoneal cells (PCs), spleen leukocytes (SLCs) and peripheral blood leukocytes (PBLCs) were investigated and associated with characteristic alterations in the glycosylation of blood serum proteins.

Materials and methods

Tumor and animal experimentation. Ascitic Ehrlich carcinoma (aneuploid strain ELD) and Cloudman S91 melanoma were obtained from the tumor strains bank of the N.N. Blokhin Russian Cancer Research Center, Moscow, Russia. Ascitic Ehrlich carcinoma cells were transplanted intraperitoneally (i.p.) to obtain ascitic tumors or by intramuscular injection (i.m.) to obtain solid tumors in the right hind limb of C57Bl/6 mice ($1x10^6$ cells/mouse diluted in 100 μl RPMI-1640 medium, Panecko, Russia). The experiments were carried out using male 2- to 3-month-old C57Bl/6 mice obtained from Stolbovaya Co. (Moscow, Russia). Cloudman S91 melanoma cells were transplanted subcutaneously (s.c.) in 200 μl RPMI-1640 medium for the estimation of tumor growth. Tumor cell suspensions for animal inoculation were prepared by mincing tumor pieces with scissors on an 80 mesh nylon screen and washing the cells through the screen with RPMI-1640 medium (1:10 w/w). The mice received standard laboratory feed and tap water *ad libitum*. All experiments were conducted in accordance with the legal regulations for animal experimentation of Russia and with official permission from the Institute of Experimental Diagnosis and Therapy of Tumors of the N.N. Blokhin Russian Cancer Research Center (7).

Tumor removal. Ascitic tumor cells were removed with ascitic fluid by means of a syringe 7 days after the inoculation of tumor cells. Solid tumor nodes (melanoma S91 or Ehrlich carcinoma) were removed by amputation of the leg under light ether anesthesia according to the protocol described by Fisher *et al* (2). The animals under narcosis were treated with ketaminum. In all experiments involving solid tumor removal, experimental and control mice were inoculated with aliquots of the same tumor cell suspension. Solid tumors were removed 14 days after the inoculation of tumor cells, once tumor volumes had reached a size of at least 1 cm^3.

Induction of resistance to tumor growth. Peritoneal cells (PCs), spleen leukocytes (SLCs) and peripheral blood leukocytes (PBLCs) were collected from operated mice at different time points (Tables I-III). PBLCs and SLCs were obtained by Ficoll gradient centrifugation (Panecko, Russia). Subsequently, $3x10^6$

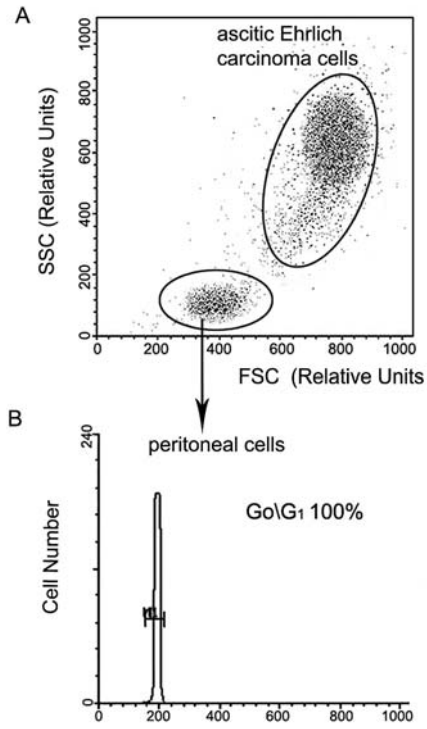

Figure 2. Flow cytometric forward and side scatter analysis of peritoneal cells and Ehrlich carcinoma cells in the ascitic fluid of mice. (A) Gate R1 shows Ehrlich carcinoma cells and gate R2, peritoneal cells. Peritoneal cells were stained with propidium iodide (B).

cells per mouse were inoculated s.c. into healthy mice. After 14 days, tumor cells were inoculated into the same mice. Each group comprised 10 animals. Statistical analysis was carried out using the Student's t-test. Data were considered significant at p-values <0.05.

Glycoprotein isolation. After the tumors reached a volume of at least 1 cm^3, glycoproteins were isolated from the blood serum of untreated healthy mice or from mice bearing i.m.-transplanted solid Ehrlich carcinoma. Serum was diluted in 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+} transferred onto PD-10 columns (Amersham Biosciences, Freiburg, Germany), and incubated for 12 h at 4˚C. The precipitate was separated by centrifugation at 10,000 x g for 20 min. Proteins were applied to ConA Sepharose columns (Pharmacia). Non-bound serum proteins were eluated by 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, while the bound proteins were eluated by a 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and 4% methyl α-d-manno-pyranoside (Sigma, USA) with the help of a GP-250 programmed gradient pump (Pharmacia). Protein elution was monitored in a flow cell at λ=280 nm. Buffer exchange from 0.05 M sodium acetate buffer to 0.1 M sodium carbonate buffer (pH 9.3) was carried out by PD-10 columns. Fluorescein 5-isothiocyanate (FITC) (Sigma, USA) was added to 0.1 M sodium carbonate buffer (pH 9.3) containing glycoproteins at 4˚C. Four hours later, the labeling of glycoproteins by FITC was stopped by 0.25 M ammonium chloride. The buffer exchange of labeled glycoproteins from 0.1 M sodium carbonate buffer (pH 9.3) to phosphate buffer solution (PBS) (pH 7.4) was carried out by PD-10 columns.

MALDI-TOF. Blood was collected from the retroorbital sinus of mice after the removal of the primary tumors at different time points (Figs. 6 and 7). The sera of animals from the treatment groups were pooled. Trypsin-type protease-cleaved peptides with 10% acetonitril were used in the peptide mixtures. Trypsin proteases were removed by an ultra-filtration PM10 membrane under air pressure (Millipore, USA). Glycopeptides from peptide mixtures were obtained by Con A Sepharose columns (Pharmacia). Glycopeptides were concentrated by the SEP-PAK C_{18}-cartridge (Millipore, USA). Mass spectra were measured by means of a MALDI-TOF Reflex I mass spectrometer (Bruker, BioSciences, Billerica, MA, USA) equipped with UV laser (336 nm). The range of positive ions was recorded from 500 to 8000 Da.

Flow cytometry. Ploidy analysis of Ehrlich carcinoma and normal peritoneal cells was carried out using flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA). After their removal from the peritoneum, Ehrlich carcinoma cells were fixed in 70% ethyl alcohol and stored at -20˚C, re-suspended in physiological solution. Normal peritoneal cells were treated likewise and served as a control.

For staining, propidium iodide (Sigma, Deisenhofen, Germany) was added at a final concentration of 1 μg/ml. Flow cytometry results were obtained from 15,000 cells collected in a gate set by forward and sideward light scatters. The results were analyzed by means of WinMDI 2.8 software. FITC-labeled glycoproteins were incubated with target cells. Peritoneal cells were washed and re-suspended in RPMI-1640 medium at a density of $3x10^6$ cells/ml. The concentration of FITC-labeled glycoproteins was equal to the concentration of these glycoproteins in blood serum.

Results

In the present study, we avoided surgically removing solid Ehrlich carcinomas, since any surgical influence is accompanied by bleeding. This also leads to an infringement of the homeostasis of the organism, which can hamper the investigation of interrelationships between the tumor and its host. Instead, we analyzed ascitic tumors by puncture using a syringe to draw ascitic fluid from the peritoneum. Cell cycle analysis of the ascitic fluids was carried out by flow cytometry 7 days after the inoculation of the tumor cells. The initial percentage of proliferating cells in ascitic fluids was ~15% (Fig. 1A). The removal of ascitic fluids caused an increase up to 30.8% in proliferating cells after 24 h (Fig. 1B). This result is comparable with the data of Fisher *et al* (2,11). After 48 h, the percentage of proliferating cells again decreased to 16% (Fig. 1C), and remained constant after 120 h (17%) (Fig. 1D).

By means of flow cytometry, it is possible to distinguish Ehrlich carcinoma cells from normal peritoneal cells. Representative histograms are shown in Fig. 2. Gate R1 contained large tumor cells (Fig. 2A), whereas gate R2 contained smaller, normal peritoneal cells (Fig. 2B). Cells in gate R2 were completely in the G0/G1 phase (100%) (Fig. 2B).

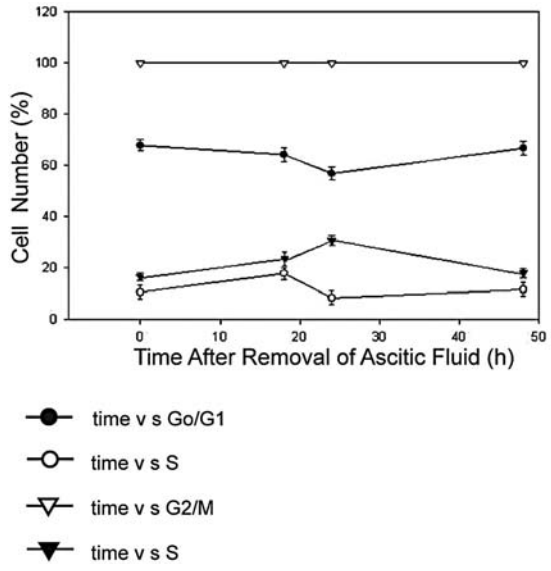

Figure 3. Influence of the removal of ascitic fluid on cell cycle distribution in Ehrlich carcinoma cells. (●) G0/G1 phase of Ehrlich carcinoma cells; (○) S phase of Ehrlich carcinoma cells; (▼) G2/M phase of Ehrlich carcinoma cells; (▽) G0/G1 phase of peritoneal cells. Representative histograms of three independent experiments are shown.

The influence of removing ascitic fluids on the cell cycle distribution of Ehrlich carcinoma and normal peritoneal cells (PCs) is shown in Fig. 3. The removal of ascitic fluid did not influence the cell cycle distribution of normal PCs, and all PCs were in the G0/G1 phase. In contrast, the cell cycle of Ehrlich tumor cells changed after the removal of ascitic fluid. An increase in the number of cells in the S phase was observed after removal of ascitic fluid, from 8.3% at 0 h to 19.1% at 18 h. Twenty-four hours after the removal of ascitic fluid, an increase in the number of cells in the G2/M phase, from 14.6% at 0 h to 31%, and a decrease in cells in the G0/G1 phase was observed.

Comparable results were found using dot plot flow cytometry (Fig. 4). Prior to the removal of ascitic fluid, the fluorescence levels of tumor cells were higher, and the cells were located in the top right quadrant of the dot plot (Fig. 4A). Twenty-four hours after the removal of ascitic fluid, the fluorescence levels of the tumor cells had decreased, and the cells were located in the bottom left quadrant of the dot plot (Fig. 4B). Similar results are also apparent in the histograms shown in Fig. 4C. The fluorescence intensity of non-tumor PCs did not change. Hence, peritoneal cells can be used as negative controls. The dynamics of changes in the average intensity of tumor cell fluorescence are shown in Fig. 4D. Significant changes in the fluorescence levels of tumor cells were observed 1 h after the removal of ascitic fluid. This decrease in fluorescence levels was no longer apparent 48 h after the removal of the ascitic fluids.

We hypothesized that the removal of a primary tumor activates a growth-inhibitory process aimed at the remaining tumor cells in the tumor-bearing host. To confirm this hypothesis, peripheral blood leukocytes (PBLCs), spleen leukocytes (SLCs) and peritoneal cells (PCs) were transferred from animals with extracted ascite tumors to healthy animals, along with Ehrlich tumor cells. A schematic of the experimental set-up is shown in Fig. 5. PBLCs, SLCs and PCs were collected

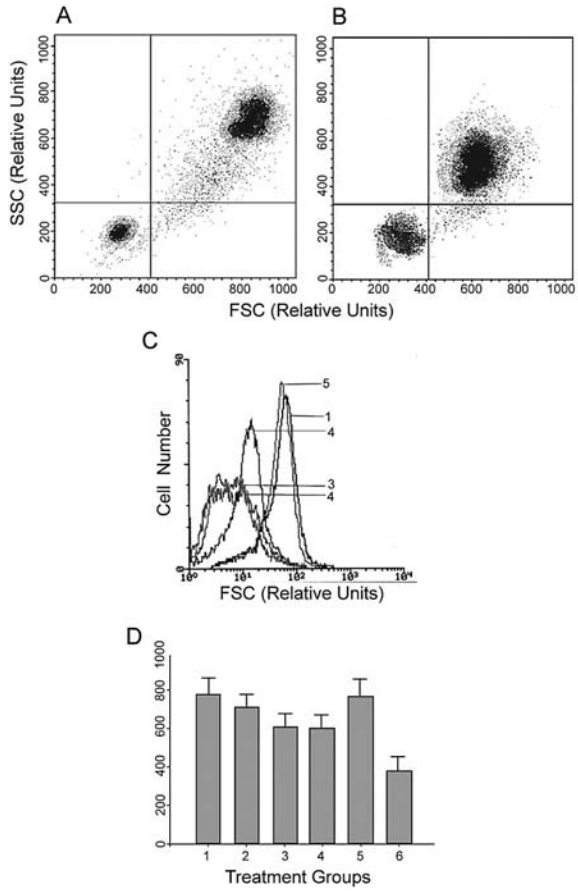

Figure 4. Flow cytometric analysis of ascitic Ehrlich carcinoma cells by forward and side scatter analysis. (A) Dot plot analysis of Ehrlich carcinoma cells from mice without the removal of ascitic fluid. Bottom left quadrant, peritoneal cells. Top right quadrant, Ehrlich carcinoma cells. (B) Dot plot analysis of Ehrlich carcinoma cells from mice 24 h after the removal of ascitic fluid. While the size of the peritoneal cells did not change , Ehrlich carcinoma cells were reduced in size after 24 h. (C) Histograms of ascitic Ehrlich carcinoma cells from mice 0 h (1), 1 h (2), 24 h (3), 48 h (4), and 120 h (5) after the removal of ascitic fluids. (D) Quantitative analysis of the influence of the removal of ascitic fluids. Mean fluorescence of ascitic Ehrlich carcinoma cells from mice 0 h (1), 1 h (2), 18 h (3), 24 h (4) and 48 h (5) after the removal of ascitic fluid. The mean fluorescence of peritoneal cells in ascitic fluids did not change significantly after the removal of the fluid (6).

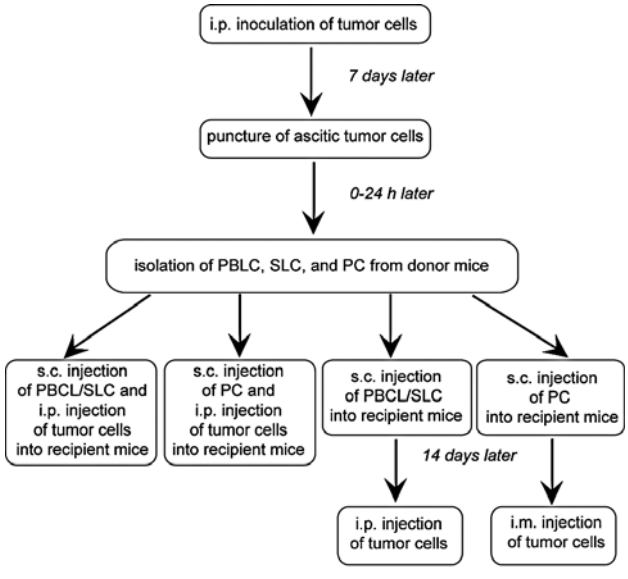

Figure 5. Schematic of the experimental set-up.

Time kinetics of donor cells to inhibit Ehrlich carcinoma cells in recipient mice after the removal of Ehrlich carcinoma from donor mice.

Group no.	Time after tumor removal (h)	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	Spleen cells	0
8.	4	SLC	0
9.	4	PBLC	0
10.	5	PC	0
11.	5	PBLC	0
12.	5	PC	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	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

Tumor cells ($1x10^6$) were injected into mice together with peripheral blood leukocytes (PBLCs), spleen leukocytes (SLCs) or peritoneal cells (PCs) ($3x10^6$). Ehrlich carcinoma cells were injected i.m. when PCs were co-applied. Ehrlich carcinoma cells were transplanted i.p. when PBLCs or SLCs were co-applied.

Table II. Time kinetics of donor cells to induce resistance to Ehrlich carcinoma in untreated healthy recipient mice after the removal of Ehrlich carcinoma from donor mice.

Group No.	Time after tumor removal (h)	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	SLC	0
8.	4	PBLC	0
9.	4	PC	0
10.	5	SLC	0
11.	5	PBLC	0
12.	5	PC	0
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	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

Peripheral blood leukocytes (PBLCs), spleen leukocytes (SLCs) or peritoneal cells (PCs) ($3x10^6$) were injected s.c., and Ehrlich carcinoma cells were injected i.m. when PCs were co-applied. Ehrlich carcinoma cells were transplanted i.p. when PBLCs or SLCs were co-applied. Fourteen days after donor cell injection, $1x10^6$ carcinoma cells were applied.

at various time points after ascitic tumor removal (0-120 h). This procedure suppressed tumor growth in 20-40% of the recipient animals when PBLCs, SLCs or PCs were collected from donor animals 6-8 h after tumor removal (Table I). A follow-up of the mice that showed resistance to tumor development after the injection of PBLCs, SLCs or PCs revealed that they maintained tumor resistance for at least 4 months, even when the number of transplanted tumor cells was increased from 10^3 to $40x10^6$ cells. This follow-up experiment is still in progress to explore the long-term effects of this phenomenon.

To ascertain whether resistance to tumor development is induced by the host or by the tumor cells co-transplanted along with PBLCs, SLCs or PCs, we performed a second set of experiments. PBLCs, SLCs or PCs were injected into mice, and Ehrlich tumor cells were inoculated 14 days later (see the scheme in Fig. 5). The results shown in Table II indicate that tumor resistance occurred within the same time frame (6-8 h) as in the previous experiment, but was more frequent (60-80% in Table II versus 20-40% in Table I). This may be evidence that the tumor-suppressive activity mediated by PBLCs, SLCs

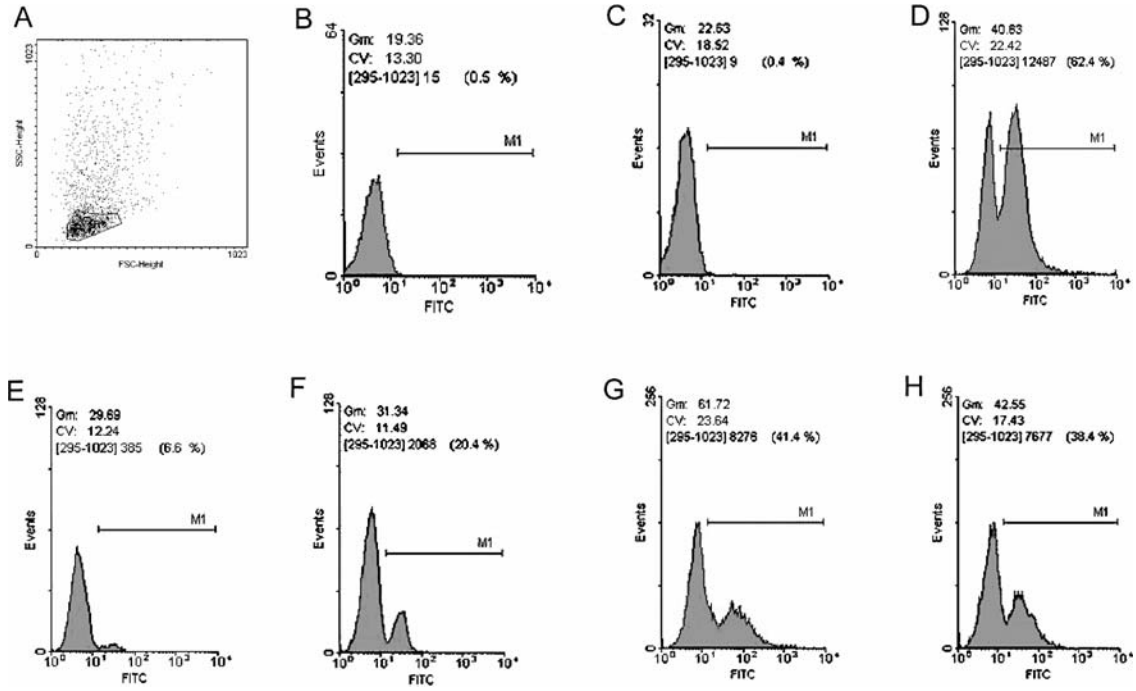

Figure 6. Flow cytometric histograms of the binding of FITC-labeled serum glycoproteins to mouse peritoneal cells. (A) Dot plot of the peritoneal cells of mice with i.m.-transplanted Ehrlich carcinoma cells. (B) Histogram of the binding of peritoneal cells from tumor-bearing mice with FITC-labeled serum glycoproteins from ascitic Ehrlich carcinoma-bearing mice without the removal of ascitic fluid (0 h). (C) Histogram of the binding of peritoneal cells from intact mice with FITC-labeled serum glycoproteins from intact mice. (D) Histogram of the binding of peritoneal cells from intact mice with FITC-labeled serum glycoproteins from ascitic tumor-bearing Ehrlich carcinoma mice without the removal of ascitic fluid. Histograms of the binding of peritoneal cells from i.m.-transplanted Ehrlich carcinoma-bearing mice with FITC-labeled serum glycoproteins from ascitic Ehrlich carcinoma-bearing mice after the removal of ascitic fluid for 1 (E), 6 (F), 18 (G) or 24 h (H).

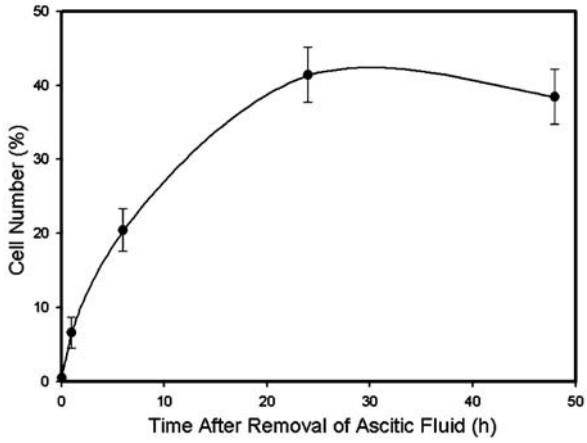

Figure 7. Quantitative analysis of the time-dependent influence of the removal of ascitic fluid on the binding of FITC-labeled serum glycoproteins from ascitic Ehrlich carcinoma cell-bearing mice after the removal of ascitic fluid injected with peritoneal cells from i.m.-transplanted Ehrlich carcinoma-bearing mice.

or PCs was induced by the host. Possibly, 6-8 h represents a necessary time frame for the recovery of animals from surgical intervention, and also for the activation of defense phenomena as observed in our set of experiments.

We speculated that post-translational protein alterations, e.g., glycosylation, may account for these short-term effects. In Fig. 6A, a flow cytometric dot plot of PCs from mice with i.m.-transplanted Ehrlich carcinoma is shown. Fig. 6B shows the histogram of the binding of PCs to FITC-labeled serum glycoproteins, which were obtained from mice with i.m.-transplanted Ehrlich carcinoma. Glycoproteins did not bind to PCs from tumor-bearing mice. Similarly, FITC-labeled serum glycoproteins from mice without tumors were not observed to bind to PCs from mice without tumors (Fig. 6C). At the same time, more than 62% of the PCs detected were immunopositive, after the incubation of PCs from mice with ascitic Ehrlich carcinoma with FITC-labeled serum glycoprotein from mice without tumors (Fig. 6D).

In Figs. 6E-H and 7, the influence of the removal of ascitic fluid on the binding of FITC-labeled serum glycoproteins is shown in a time-dependent manner. Initially (0 h) the percentage of positive cells was zero or very low. One hour after the removal of ascitic fluid, the percentage of positive cells increased up to 6% and, after 7 h, up to 21%. A maximum of ~40% was reached 24 h after the removal of the ascitic fluid. These data show that changes in PBLCs, SLCs and PCs, as well as in serum glycoprotein immunoreactivity, occurred after 6-8 and 18 h, respectively.

Glycopeptides of blood serum glycoproteins were obtained by hydrolysis with trypsin and subsequent purification. In Fig. 8A, a mass spectrum of glycopeptides from the digested blood serum glycoprotein of healthy male mice is shown. The mass spectra of healthy mice differed from the mass spectra of tumor-bearing animals in glycopeptides of high molecular weight (m/z 4531.96 and m/z 4819.394). In tumor-bearing mice (Fig. 8B), the spectrum of glycopeptides differed from the spectra of glycopeptides obtained from mice 8 and 24 h after the removal of ascitic fluid (Fig. 8C and D). The spectra of glycopeptides illustrate that the process of glycosylation alterations is rapid. The velocity of this process might account for the results described above.

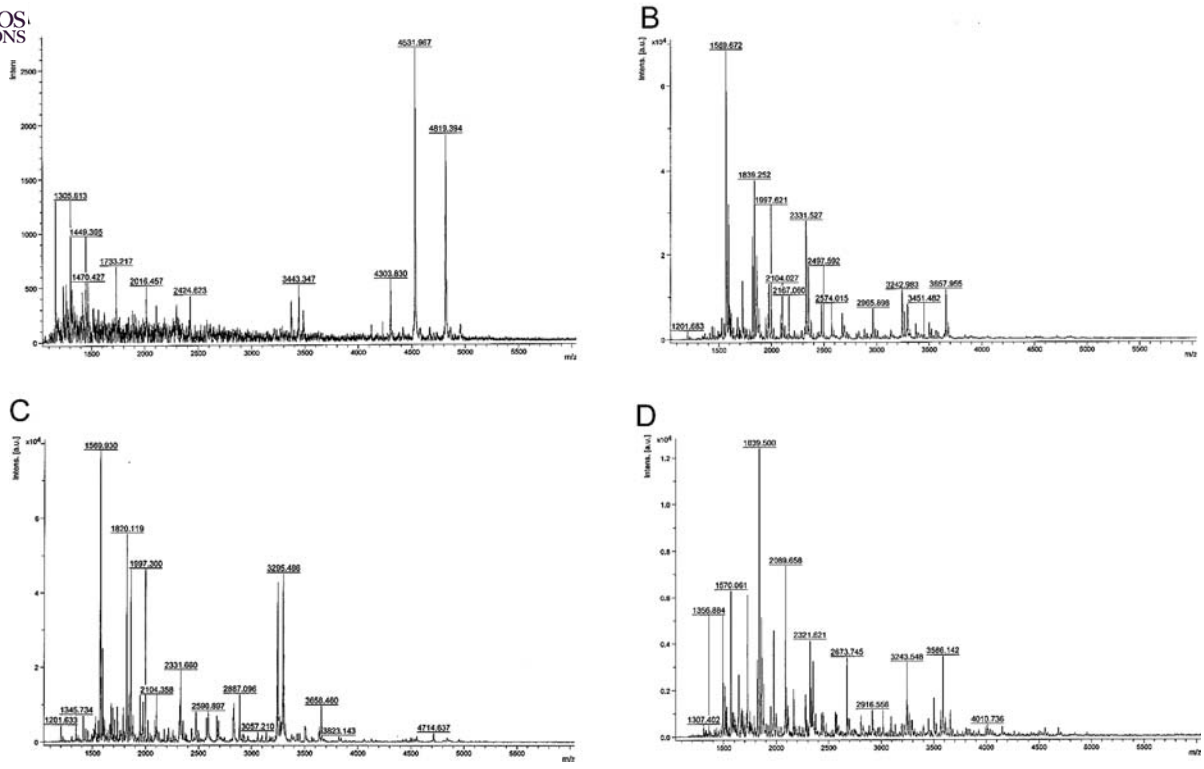

Figure 8. Mass spectrometry profiles of serum trypsin-type protease-cleaved glycopeptides. Trypsin-type protease-cleaved glycopeptides were obtained from the serum proteins of untreated control C57bl/6 (A) and Ehrlich carcinoma-bearing C57bl/6 mice 0 (B), 8 (C) or 24 h (D) after removal of ascitic fluid.

Table III. Influence of the modes of application of donor cells on the growth of Cloudman S91 melanoma in mice.

Group no.	Time after tumor removal (h)	Cell type	No. of donor cell injections	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	40.0±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

Peripheral blood leukocytes (PBLCs), spleen leukocytes (SLCs), or peritoneal cells (PCs) ($3x10^6$) were subcutaneously injected. Single, double, or triple injections were performed. Fourteen days after donor cell injection, $1x10^6$ Cloudman melanoma cells were subcutaneously applied.

We observed this phenomenon not only in Ehrlich carcinoma, but also in Cloudman S91 melanoma (Table III). Recipient mice injected with PBCLs, SLCs or PCs from operated donor animals were revealed to have less frequently occuring tumors when these cells were collected 6-8 h after surgery. The percentage of mice resistant to tumor development was increased when the cells were injected repeatedly. Single injection of these cells resulted in 20% of the mice exhbiting resistance to melanoma development. Double transplantation increased the number of resistant mice up to 40-70%, while after triple transplantation resistance was found in up to 100% of the mice. These results strongly suggest that tumor-suppressive effects are conferred by the host organism.

Discussion

In the present investigation, we found that the removal of Ehrlich carcinoma cells growing as ascitic tumors increased the fraction of proliferating tumor cells. The maximum effect was observed 24 h after tumor removal. This result is in good accordance with data in the literature, showing increased proliferative activity in the remaining tumor cells after the surgical removal of solid tumors (8,9).

There are two possible explanations for this phenomenon. One asserts that tumor cells release a certain factor, which is activated after the removal of the tumor. This hypothesis was first proposed by Weiss in the 1950s (10). The chemical nature of this factor is unknown.

We favor a second 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 the removal of the tumor, the concentration of this factor drastically increases. This increased concentration in blood serum leads to a negative feedback loop, resulting in a subsequent decrease of the factor in serum. However, the question as to why such a hypothetical negative feedback loop is activated remains.

One possible answer to this question is provided by our flow cytometry data. Using forward and side scattering, we observed a change in the size of tumor cells after the partial removal of ascitic fluid. This change in tumor cell size may be associated with a change in the interaction of these cells with molecules in the ascitic fluid. This assumption is important in the postulation of 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 in these chemical groups might lead to the dissociation of these molecules from the surface, or to the formation of molecular complexes. Complexes may be formed between biomacromolecules, between biomacromolecules and cells, and as conglomerates of cells. The formation of cell conglomerates may pose a danger to an organism, as it causes the development of the so-called 'sludge' syndrome. This syndrome occurs, for example, after the dehydration of an organism or following sepsis. Dehydration causes an increase in the erythrocyte number in blood. As a result, erythrocytes form monetary column-formed structures (11,12).

Therefore, in the present study, we examined the interaction of blood serum glycoproteins and peritoneal cells in mice. Our results indicate an increased binding of glycoproteins with peritoneal cells after the removal of ascitic fluid. The formation of conglomerates of the remaining tumor cells in ascitic fluid was observed by visual inspection. Hence, it is worth speculating that the partial removal of ascitic fluid incites a condition in the remaining cells similar to the 'sludge' syndrome. Such a condition might be harmful to the tumor-bearing host, and could lead to a switch to compensatory mechanisms. As a result, the affinity of biomacromolecules to tumor and peritoneal cells might be changed. Changes in the affinity of proteins to cells can be measured as alterations in their glycosylation pattern. We demonstrated that the glycosylation of blood serum proteins changed within 6 h of the removal of ascitic fluid. It is reasonable to assume that lymphocytes can carry out protein glycosylation reactions by means of the recognition and dissociation of formed complexes, which can then be released into biological fluids, such as ascitic fluid.

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 (13-20). A factor appears in blood serum which enhances cell proliferation as early as 12 h after small bowel resection (21). There are several indications in the literature that a serum factor causes an increase in vascular endothelial growth factor (VEGF) after organ resection (22,23). This factor is not VEGF, since VEGF does not display 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 (24).

The occurrence of lipomatosis after cosmetic surgery also supports our view of complex balances (25-28). Goshtasby *et al* (29) described a case of isolated symmetrical lipomatosis of soft tissue overlying the trochanters that developed a recurrence after liposuction treatment. 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.

Another line of evidence comes from studies by Scheiffarth *et al* (30) and Warnatz *et al* (31). 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 abovementioned studies (30,31), 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 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.

Taking our results and the data in the literature into account, we can draw the following conclusions: i) the removal of Ehrlich carcinoma accelerated tumor growth for up to 24 h following surgery. Subsequently, accelerated tumor growth was inhibited. This is not a tumor-specific phenomenon associated solely with Ehrlich carcinoma, as we also observed it in

 n S91 melanoma. ii) An inhibition of tumor growth by donor cells (PBLCs, SLCs or PCs) can be transferred from operated donor animals to recipient animals. iii) This mechanism of tumor growth inhibition may be related to a tumor-specific factor in blood serum. iv) Blocking this factor may slow down the proliferation of tumors. v) This phenomenon is associated with characteristic alterations in the glycosylation of blood serum proteins. Research is currently being conducted to identify and characterize this factor.

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