

## Identification of Serpin ( $\alpha$ -1-Antitrypsin) as Serum Growth Inhibitory Factor in Murine Ehrlich Carcinoma by Proteomics

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**Abstract.** *It is well established that serum factors play a role in relapse of tumor diseases after removal of the primary tumor. The molecular nature of these factors and their mechanism of action remain unknown. We focused on host-related mechanisms to identify tumor-specific serum factors of mice bearing Ehrlich carcinoma, which have the potential to confer resistance towards tumor development. An experimental model was used, where we incubated isolated immune cells (peritoneal cells (PCs) and splenic lymphocytes (SLCs)) in vitro with blood serum or ascitic fluid from tumor-bearing mice. Mice inoculated with PCs or SLCs previously incubated for 7 h with ascitic fluid from tumor-bearing mice did not develop tumors at a frequency of 93.1±5.7% (inoculation of tumor cells after two weeks) and 100% (inoculation of tumor cells three months later). This indicates that mice developed resistance towards tumor development. By fractionation of ascitic fluid and (LC/MS-MS)-driven profiling of serum proteins, we identified serpin ( $\alpha$ -1-antitrypsin), which was missing from the PC-incubated fraction, indicating that this protein was bound to PCs and, thereby, purged from the protein fraction. In parallel, cathepsin L1 appeared after incubation with PCs. 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. Furthermore, serpins may protect parasites against the immune systems of the host. Taken together, it can be*

*hypothesized that serpin represents a tissue- and tumor-specific anti-proteinase.*

It is well established that serum factors play a role in relapse of tumor diseases after removal of the primary tumor (1-6). The molecular nature of these factors and their mechanism of action for tumor growth, however, remain unknown. Recent data show that not only tumors, but also normal tissues of host possess the ability for compensatory growth. An example is lipomatotic growth of fatty tissue after cosmetic surgery (7-10). Lipomatosis is the growth of a fatty tissue in trochanters, heart and kidneys after liposuction (11). The presence of host-related factors may explain the regulation of both the malignant tumor growth and benign tissue growth, as in the case of lipomatosis.

Our own research efforts focus on such host-related mechanisms, which regulate the growth of Ehrlich carcinoma cells in mice. Previously, we found that complexes of albumin and hemoglobin in blood serum induced apoptosis of Ehrlich carcinoma *in vivo* leading to cure of mice from their tumor (12). Transferring immune cells from mice whose tumors had been removed to other mice prevented tumor growth. This effect was time dependent. Blood serum taken 6-8 h after removal of the tumor revealed the strongest tumor-inhibitory effect. This did not represent an artificial effect solely seen in Ehrlich carcinoma, since similar results were obtained with Cloudman S-91 melanoma (13). Therefore, we hypothesized the existence of factors in blood serum which confer resistance towards tumor development. Our results also imply that the hypothesis of Fisher and co-workers (2) on accelerated tumor growth after removal of the primary tumor may be erroneous. This may explain why previously hypothesized growth-stimulatory factors in serum following primary tumor removal (2) have never been identified.

By contrast, we have assumed that factors affecting tumor growth may exist in the tumor-bearing host and that these

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factors do not only reveal growth-regulatory function for tumors, but also for healthy organs and tissues of an organism. Possibly, such factors are produced and subsequently absorbed by the host. Removal of target cells, e.g. tumor cells, might lead to reduced absorption rates of these factors. Hence, removal of tumor cells might be accompanied by an increase of the concentration of these factors in blood serum.

We have shown that increasing the levels of an hitherto uncharacterized factor induced a negative feedback mechanism, which inhibited the development of this factor by the host (13). 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. It has also recently been shown by us that glycoproteins, peripheral blood leukocytes, splenic leukocytes, and peritoneal cells participate in the regulation of this balance (13). 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 (13).

The aim of the present 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.

## Materials and Methods

**Tumor and animal experimentation.** Ascitic Ehrlich carcinoma (aneuploid strain ELD) was obtained from the bank of tumor strains 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 to obtain solid tumors by intramuscular injection (*i.m.*) into the right hind limb of C57Bl/6 mice ( $1 \times 10^6$  cells/mouse diluted in 100  $\mu$ l RPMI-1640 medium; Panecko, Russia). The experiments were carried out using 2- to 3-month-old C57Bl/6 male mice. The animals were obtained from Stolbovaya Company (Moscow, Russia). The mice received standard laboratory feed and tap water *ad libitum*. All experiments were carried out in accordance with the legal regulations for animal experimentation in Russia and with official permission of the Institute of Experimental Diagnosis and Therapy of Tumors of the N. N. Blokhin Russian Cancer Research Center (14).

**Induction of resistance towards tumor growth.** Peritoneal cells (PCs) and splenic cells (SLCs) were collected from intact mice. The technique of SLCs isolation leads to destruction of the spleen and to a certain extent also of SLCs. Debris of destroyed SLCs will be in the medium, complicating protein identification. Since the isolation of PCs is gentle, we isolated this type of cells.

Ascitic fluid was collected from mice with ascitic Ehrlich carcinoma 10 days after tumor cells injection. Proteins of ascitic fluid were separated into fractions by ultrafiltration membranes under air pressure with nominal molecular weight limit (NMWL) of 300 kDa (fraction 1), 100 kDa (fraction 2), 50 kDa (fraction 3) and 10 kDa (fraction 4) (Millipore, USA). Proteins were diluted in RPMI-1640 medium and transferred to PD-10 columns (Amersham Biosciences, Freiburg, Germany). Cell and protein fractions were co-incubated for different times, 7 h for experimental groups and 4 h for control groups at 37°C and 5% CO<sub>2</sub> (Table I). Duration of cell incubation with fractions of proteins has been determined and previously described (13). Subsequently,  $3 \times 10^6$  cells per mouse were subcutaneously inoculated into healthy mice. After 14 days,  $1 \times 10^6$  tumor cells were inoculated into these mice intraperitoneally. (Each group included 10 animals. Statistical calculations were made using Student's *t*-test. The data were considered as significant at *p*-values below 0.05.

**Affinity chromatography.** Ascitic fluid from tumor-bearing mice 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<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> transferred to PD-10 columns (Amersham Biosciences), and left for 12 h at 4°C. The precipitate was separated by centrifugation at 10,000  $\times$ g for 20 min. Proteins were applied to ConA Sepharose columns (Pharmacia). Non-bound serum proteins were eluted by 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>, while the bound proteins were eluted by an 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup> and 2% saccharose (fraction 5) and then were eluted by an 0.05 M sodium acetate buffer (pH 6.0, 0.01% sodium azide) containing 0.25 M sodium chloride and 1 mM Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup> and 4% methyl  $\alpha$ -D-manno-pyranoside (fraction 6) (Sigma, USA) with the help of a GP-250 programmed gradient pump (Pharmacia). Protein elution was monitored in a flow cell at  $\lambda=280$  nm.

**Gel-filtration.** Fraction 5 was additionally purified by a gel-filtration using Superdex G-75 (GE Health Care, USA) to collect major protein peaks.

**Protein gel electrophoresis.** Protein samples were separated by SDS/PAGE on a 12.5% gel and stained with Coomassie blue according to Laemmli (15). Samples subjected to SDS/PAGE were solubilized in sample buffer, containing 63 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and 30 M bromophenol blue. 2-Mercaptoethanol 5% (v/v) was conditionally added or omitted in the sample buffer. Unless stated otherwise, 12.5% acrylamide gels with a bisacrylamide/acrylamide ratio of 0.8:30 were used. Samples were applied in quantities of 10 and 50  $\mu$ g protein/lane to evaluate all components of protein complexes.

**Mass spectrometry.** Samples were prepared according to Laemmli with some modifications (15). After SDS/PAGE (8-20% gradient gel), the gel was stained with Coomassie R-250 and protein bands were excised and cut into 1 $\times$ 1 mm pieces. A piece of gel was washed twice for 5 min in 70  $\mu$ l 50% 200 mM NH<sub>4</sub>HCO<sub>3</sub>-50% acetonitrile mixture (v/v) and placed in 70  $\mu$ l acetonitrile for 15 min. Acetonitrile was then removed, and the gel was dried using a SpeedVac for 20 min. The dried gel piece was put in 3  $\mu$ l trypsin solution (15 ng/ $\mu$ l) in 50 mM

Table I. 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.

| N  | Fraction of ascitic fluid   | Number of resistant mice compared to all mice 14 days after donor cells injection <sup>#</sup> | 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 <sup>#</sup>   |
|    | 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 <sup>#</sup>   |
|    | 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 <sup>#</sup> /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   |  |

Peritoneal cells (PCs) ( $3 \times 10^6$  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,  $1 \times 10^6$  Ehrlich carcinoma cells were intraperitoneally applied. 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 ( $3 \times 10^6$  cells). <sup>#</sup>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.

$\text{NH}_4\text{HCO}_3$ , kept on ice for 30 min, and then incubated overnight at 37°C. The next day, 6  $\mu\text{l}$  50% acetonitrile in 0.1% trifluoroacetic acid (TFA) were added to the gel, and the gel was incubated for 2 h. After incubation, 2  $\mu\text{l}$  of the above gel solution were taken and mixed with matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in 50% acetonitrile–0.1% TFA, v/v) and applied to mass spectrometry (MS). Mass spectra were acquired using an MALDI time-of-flight (TOF)/MS (Ultraflex TOF/TOF; Bruker Daltonik GmbH, Bremen, Germany) equipped with 337 nm nitrogen laser in reflector mode in the *m/z* range 800–5000 Da. The data were processed using Bruker Daltonics Flex Analysis 2.2 software, and peaks of trypsin fragments contained in samples were used for calibration. The following search parameters were used: accuracy of mass determination 100 ppm, SwissProt protein sequence Database, Rodentia taxon (rodent), one missed cleavage, possible methionine oxidation. If the value of Score parameter calculated for each protein exceeded 70, identification was accepted as reliable. The method for calculation of the Score parameter is described at [http://www.matrixscience.com/help/scoring\\_help.html](http://www.matrixscience.com/help/scoring_help.html).

**Liquid chromatography-electrospray injection (LC-ESI) MS/MS.** Experiments were carried out using an LC-MS system, consisting of an Agilent 1200 Series HPLC-Chip (Agilent Technology) combined with an Agilent 6520 Accurate-Mass Q-TOF LC/MS system (Agilent Technology). Before and after incubation proteins of fraction 5 with PCs, proteins of fraction 5 were additionally purified by gel-filtration with Superdex G-75 to collect major protein peaks.

**Ion-exchange chromatography.** Samples of fraction 5 before and after incubation with PCs were applied to Sepharose QFF columns

equilibrated with 20 mM Tris/HCl buffer (pH 7.4). Fractions were eluted from the column with a linear gradient of 0–0.35 M NaCl in the equilibration buffer, in order to increase the sensitivity of LC-MS. All proteins identified are listed in Table II. Comparative constituents of fractions containing  $\alpha$ -1-antitrypsin are shown in Tables III and IV.

**HPLC-Chip.** Protein ID chip with 150 $\times$ 0.075 mm analytical column and 40 nl enrichment column were used. The experimental conditions were as follows: Sample load: 5  $\mu\text{l}$  of peptide solution obtained from in-gel trypsin digestion of proteins; flow: 300 nl/min analytical pump, 3  $\mu\text{l}/\text{min}$  loading pump; mobile phases A: 5% acetonitrile, 0.1% formic acid (FA), B: 90% acetonitrile, 0.1% FA; gradients: 0% B to 50% B at 50 min, then 80% B at 60 min until 65 min, then 0% B at 65.1 min; stop time: 70 min and 10 min equilibration time. The analytes were trapped in 100% solvent A.

**Q-TOF MS.** The conditions were: drying gas: 4 l/min, 325°C; skimmer: 65 V; fragmentor: 175 V; collision energy: slope 3.7 V, offset 2.5 V; MS scan range and rate: 300–2000 at 3 Hz; MS/MS scan range and rate: 50–3000 at 3 Hz; autoMS/MS: 3 precursors, active exclusion on with 1 repeat and release after 0.3 min; preferred charge state: 2, 3, >3, unknown.

**Database searches.** Protein database searches were performed with Spectrum Mill MS Proteomics Workbench Rev A.03.03.084 SR4 (licensed to Kurchatov Institute, Moscow, Russia). The SwissProt database (Rodentia taxon) was used with trypsin specificity, one missed cleavage, 50% minimum scored peak intensity, and dynamic peak threshold.

Table II. Identification of proteins in tumor resistance-conferring protein fractions of ascitic fluid from Ehrlich carcinoma-bearing mice by LC/MS.

|     | Protein name  | Protein MW (Da) | Database accession# | Protein pI |
|-----|---|-----------------|---------------------|------------|
| 1.  | Haptoglobin   | 38752.5         | Q61646              | 5.88       |
| 2.  | Clusterin   | 51655.9         | Q06890              | 5.47       |
| 3.  | H-2 class I histocompatibility antigen, Q10 alpha chain 1 | 37251.2         | P01898              | 5.13       |
| 4.  | Hemoglobin subunit beta-2                                 | 15878.3         | P02089              | 7.85       |
| 5.  | Inter-alpha-trypsin inhibitor heavy chain H3              | 99366.2         | Q61704              | 5.70       |
| 6.  | Carboxypeptidase N subunit 2                              | 60479.5         | Q9DBB9              | 5.53       |
| 7.  | Alpha-1-antitrypsin 1-1 Serpina1a                         | 46002.8         | P07758              | 5.44       |
| 8.  | Kininogen-1   | 73102.5         | O08677              | 6.05       |
| 9.  | Angiotensinogen   | 51990.7         | P11859              | 5.26       |
| 10. | Inter-alpha-trypsin inhibitor heavy chain H2              | 105928.4        | Q61703              | 6.82       |
| 11. | Plasma kallikrein   | 71369.0         | P26262              | 8.40       |
| 12. | Heat-shock cognate 71 kDa protein                         | 70871.4         | P63017              | 5.38       |
| 13. | Serine protease inhibitor A3K                             | 46880.2         | P07759              | 5.05       |
| 14. | Complement factor B                                       | 85005.0         | P04186              | 7.18       |
| 15. | Fibrinogen gamma chain                                    | 49391.7         | Q8VCM7              | 5.54       |
| 16. | Plasminogen   | 90782.4         | P20918              | 6.20       |
| 17. | Apolipoprotein A-I  | 30587.6         | Q00623              | 5.65       |
| 18. | Serotransferrin   | 76724.3         | Q92111              | 6.94       |
| 19. | Platelet glycoprotein V                                   | 63468.2         | O08742              | 9.23       |
| 20. | Fibrinogen beta chain                                     | 54753.0         | Q8K0E8              | 6.68       |
| 21. | Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA 2       | 73276.2         | P45700              | 6.34       |
| 22. | Sulfhydryl oxidase 1                                      | 82785.8         | Q8BND5              | 6.73       |
| 23. | Complement component C8 beta chain 2                      | 66229.8         | Q8BH35              | 8.07       |
| 24. | Hemopexin   | 51341.1         | Q91X72              | 7.92       |
| 25. | Fetuin-B  | 42713.0         | Q9QXC1              | 6.17       |
| 26. | Complement component C8 alpha chain 3                     | 66080.7         | Q8K182              | 6.13       |
| 27. | Inter-alpha-trypsin inhibitor heavy chain H1              | 101083.4        | Q61702              | 6.50       |
| 28. | Inter-alpha-trypsin inhibitor heavy chain H3              | 99366.2         | Q61704              | 5.70       |
| 29. | Inter-alpha-trypsin inhibitor heavy chain H2              | 105928.4        | Q61703              | 6.82       |
| 30. | Ig mu chain C region membrane-bound form 4                | 52527.1         | P01873              | 5.98       |
| 31. | Complement component C9                                   | 62002.7         | P06683              | 5.56       |
| 32. | Vitronectin   | 54849.3         | P29788              | 5.67       |
| 33. | Complement C1s-A subcomponent                             | 76858.0         | Q8CG14              | 4.97       |
| 34. | Alpha-1-antitrypsin 1-2 Serpina1b                         | 45974.9         | P22599              | 5.32       |
| 35. | Liver carboxylesterase N                                  | 61172.4         | P23953              | 5.11       |
| 36. | Thyroxine-binding globulin Serpina7 5                     | 47052.5         | P61939              | 6.07       |
| 37. | Pigment epithelium-derived factor                         | 46234.3         | P97298              | 6.48       |
| 38. | Insulin-like growth factor-binding protein complex acid 6 | 66960.3         | P70389              | 6.13       |
| 39. | Serum albumin   | 68678           | P07724              | 5.75       |
| 40. | Zinc-alpha-2-glycoprotein                                 | 35256.0         | Q64726              | 5.83       |

*Exhaustion of biological activity of fraction 5.* The interaction of FITC-labeling glycoproteins of serum blood with PCs has previously been described. It has been shown that removal ascitic liquids (a primary tumor) of mice is accompanied by an increase in linkage FITC-labeling glycoproteins of serum with PCs. This change of interaction of serum glycoproteins and PCs was tumor-specific and is caused by tumor removal in mice. To simulate this phenomenon *in vitro*, PCs were isolated from mice without tumor, and serum glycoproteins from mice with tumor. As this animal system is syngenic all other antigens should be identical (13).

PCs from intact mice ( $5 \times 10^6$  cells/ml) were incubated with medium containing proteins of fraction 5 for 4 h at 37°C and 5% CO<sub>2</sub>. Proteins of fraction 5 were obtained as described above. Proteins were then diluted in RPMI-1640 medium transferred to PD-10 columns (Amersham Biosciences). Volumes of medium were

approximately five time less than volumes of the ascitic fluids which these proteins were derived from. Cells were then removed by centrifugation for 15 min at 800 ×g. The same procedure was applied for spleen cells. After the incubation of cells with medium, they were filtered through 0.22 μm membrane. The medium was subjected to a Superdex G-75 column for removal of proteins with different sizes than proteins of fraction 5. The exhausted fraction 5 lost the ability to induce resistance to Ehrlich carcinoma growth in mice.

## Results

The experimental design is shown in Figure 1. The results of the experiments are presented in Table I. Mice inoculated with PC previously incubated for 7 h with ascitic fluid did not



Table III. Identification of proteins from fraction 5 which were eluted during ion-exchange chromatography together with serpin ( $\alpha$ -1-antitrypsin) before incubation with peritoneal cells by LC/MS.

| Run # | Run name | Group (#) | Spectra (#) | Distinct peptides (#) | Distinct summed MS/MS search score | % AA coverage | Mean peptide spectral intensity | Protein MW (Da) | Protein pI | Database accession # | Protein name   |
|-------|----------|-----------|-------------|-----------------------|------------------------------------|---------------|---------------------------------|-----------------|------------|----------------------|--|
| 2     | D2       | 1         | 10          | 8                     | 148.87                             | 28            | 5.48e+005                       | 45974.9         | 5.32       | P22599               | $\alpha$ -1-Antitrypsin 1-2                                |
| 2     | D2       | 2         | 6           | 5                     | 100.61                             | 7             | 1.87e+005                       | 38752.5         | 5.88       | Q61646               | Haptoglobin  |
| 2     | D2       | 3         | 7           | 5                     | 57.10                              | 12            | 3.23e+005                       | 37251.2         | 5.13       | P01898               | H-2 class histocompatibility antigen, Q10 $\alpha$ chain I |
| 2     | D2       | 4         | 9           | 3                     | 49.37                              | 4             | 2.18e+005                       | 73102.5         | 6.05       | O08677               | Kininogen-1  |
| 2     | D2       | 5         | 2           | 2                     | 45.85                              | 5             | 2.73e+005                       | 105928.4        | 6.82       | Q61703               | Inter- $\alpha$ -trypsin inhibitor heavy chain H2          |
| 2     | D2       | 6         | 2           | 2                     | 44.13                              | 8             | 1.01e+005                       | 61172.4         | 5.11       | P23953               | Liver carboxylesterase N                                   |
| 2     | D2       | 7         | 2           | 2                     | 38.56                              | 5             | 8.94e+004                       | 51990.7         | 5.26       | P11859               | Angiotensinogen  |
| 2     | D2       | 8         | 2           | 2                     | 36.02                              | 5             | 1.55e+005                       | 99366.2         | 5.70       | Q61704               | Inter- $\alpha$ -trypsin inhibitor heavy chain H3          |
| 2     | D2       | 9         | 2           | 2                     | 32.07                              | 4             | 1.55e+005                       | 60479.5         | 5.53       | Q9DBB9               | Carboxypeptidase N subunit 2                               |
| 2     | D2       | 10        | 2           | 2                     | 27.75                              | 21            | 3.00e+004                       | 15878.3         | 7.85       | P02089               | Hemoglobin subunit $\beta$ -2 OS                           |
| 2     | D2       | 11        | 2           | 2                     | 22.42                              | 1             | 1.33e+005                       | 101083.4        | 6.50       | Q61702               | Inter- $\alpha$ -trypsin inhibitor heavy chain H1          |
| 2     | D2       | 12        | 2           | 2                     | 20.46                              | 5             | 7.68e+004                       | 46234.3         | 6.48       | P97298               | Pigment epithelium-derived factor                          |
| 2     | D2       | 13        | 2           | 2                     | 18.71                              | 3             | 1.02e+005                       | 41302.1         | 5.96       | P01901               | H-2 class I histocompatibility antigen, K-B $\alpha$ chain |
| 2     | D2       | 14        | 2           | 2                     | 16.69                              | 3             | 1.50e+005                       | 30587.6         | 5.65       | Q00623               | Apolipoprotein A-I   |
| 2     | D2       | 15        | 2           | 1                     | 15.64                              | 2             | 5.09e+004                       | 66229.8         | 8.07       | Q8BH35               | Complement component C8 $\beta$ chain                      |
| 2     | D2       | 16        | 1           | 1                     | 14.92                              | 3             | 1.12e+005                       | 49391.7         | 5.54       | Q8VCM7               | Fibrinogen $\gamma$ chain                                  |
| 2     | D2       | 17        | 1           | 1                     | 14.69                              | 3             | 5.22e+004                       | 54753.0         | 6.68       | Q8K0E8               | Fibrinogen $\beta$ chain                                   |
| 2     | D2       | 18        | 1           | 1                     | 12.75                              | 5             | 1.98e+005                       | 51655.9         | 5.47       | Q06890               | Clusterin  |

The isolation of serpin was performed by the method of Mistry *et al.* (22).

develop tumors at a frequency of 9 out of 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 present work, the infringement of balance between tumor and host was simulated *in vitro* by incubation of PCs and SLCs from mice without tumor with ascitic liquid or its fractions from tumor-bearing mice. Table I 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 intraperitoneal injection of  $1 \times 10^6$  tumor cells per

mouse, tumor growth was not detected at least for one year. By contrast, resistance towards tumor formation was not observed in the control group and the 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 eluted by methylmannopyranoside (fraction 6). Corresponding controls were prepared for all six fractions. As shown in Table I, fractions 1 and 2 weakly prevented tumor formation in mice. Only 3 out of 10 mice were resistance to tumor growth. This resistance towards tumor growth was short. A repeated injection of ascitic cells three months later resulted in tumor growth in three mice. The tumor growth rate

Table IV. Identification of proteins from fraction 5, which were eluted during ion-exchange chromatography together with serpin ( $\alpha$ -1-antitrypsin) after incubation with peritoneal cells by LC/MS.

| Run # | Run name | Group (#) | Spectra (#) | Distinct peptides (#) | Distinct summed MS/MS search score | % AA coverage | Mean peptide spectral intensity | Protein MW (Da) | Protein pI | Database accession # | Protein name   |
|-------|----------|-----------|-------------|-----------------------|------------------------------------|---------------|---------------------------------|-----------------|------------|----------------------|--|
| 1     | D1       | 1         | 13          | 12                    | 208.79                             | 9             | 1.18e+006                       | 38752.5         | 5.88       | Q61646               | Haptoglobin  |
| 1     | D1       | 2         | 4           | 9                     | 164.28                             | 12            | 1.09e+005                       | 51655.9         | 5.47       | Q06890               | Clusterin  |
| 1     | D1       | 3         | 10          | 9                     | 163.05                             | 12            | 2.41e+005                       | 37251.2         | 5.13       | P01898               | H-2 class I histocompatibility antigen, Q10 $\alpha$ chain 1 |
| 1     | D1       | 4         | 5           | 5                     | 55.62                              | 28            | 1.12e+005                       | 15878.3         | 7.85       | P02089               | Hemoglobin subunit $\beta$ -2                                |
| 1     | D1       | 5         | 5           | 5                     | 55.31                              | 6             | 2.83e+005                       | 99366.2         | 5.70       | Q61704               | Inter- $\alpha$ -trypsin inhibitor heavy chain H3            |
| 1     | D1       | 6         | 4           | 4                     | 55.21                              | 7             | 1.61e+005                       | 60479.5         | 5.53       | Q9DBB9               | Carboxypeptidase N subunit 2                                 |
| 1     | D1       | 7         | 3           | 3                     | 46.13                              | 4             | 1.81e+005                       | 73102.5         | 6.05       | O08677               | Kininogen-1  |
| 1     | D1       | 8         | 3           | 2                     | 44.67                              | 5             | 5.80e+005                       | 51990.7         | 5.26       | P11859               | Angiotensinogen  |
| 1     | D1       | 9         | 2           | 2                     | 31.92                              | 3             | 1.16e+005                       | 105928.4        | 6.82       | Q61703               | Inter- $\alpha$ -trypsin inhibitor heavy chain H2            |
| 1     | D1       | 10        | 3           | 2                     | 30.66                              | 3             | 1.14e+005                       | 71369.0         | 8.40       | P26262               | Plasma kallikrein (Mus musculus)                             |
| 1     | D1       | 11        | 2           | 2                     | 25.02                              | 4             | 5.98e+004                       | 70871.4         | 5.38       | P63017               | Heat-shock cognate 71 kDa protein                            |
| 1     | D1       | 12        | 2           | 1                     | 22.29                              | 5             | 1.04e+005                       | 46880.2         | 5.05       | P07759               | Serine protease inhibitor A3K                                |
| 1     | D1       | 13        | 2           | 1                     | 21.60                              | 4             | 1.16e+005                       | 42051.3         | 5.23       | P68134               | Actin, $\alpha$ skeletal muscle                              |
| 1     | D1       | 14        | 1           | 1                     | 15.99                              | 1             | 5.21e+004                       | 85005.0         | 7.18       | P04186               | Complement factor B  |
| 1     | D1       | 15        | 1           | 1                     | 15.22                              | 3             | 9.88e+004                       | 49391.7         | 5.54       | Q8VCM7               | Fibrinogen $\gamma$ chain                                    |
| 1     | D1       | 16        | 1           | 1                     | 14.93                              | 0             | 5.59e+004                       | 90782.4         | 6.20       | P20918               | Plasminogen  |
| 1     | D1       | 17        | 1           | 1                     | 14.88                              | 3             | 8.01e+004                       | 30587.6         | 5.65       | Q00623               | Apolipoprotein A-I   |

The isolation of serpin was performed by the method of Mistry *et al.* (22).

in these mice was much less than in the control group. In the 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 out of ten mice which received fraction 5 were resistant to tumor growth after 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. As can be seen in Table II, 40 proteins were identified in the activated protein fractions from tumor-

bearing mice, which were not found in the control samples from untreated animals.

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 ( $\alpha$ -1-antitrypsin) was absent from the PC-incubated fraction, indicating that this protein was bound to PCs and, thereby, purged from the protein fraction (Tables III and IV). The mass spectrum of serpin is shown in Figure 2.

This result obtained by LC/MS-MS was confirmed by gel electrophoresis and MALDI-TOF analyses. Fraction 5 revealed two major bands (A, B) with similar intensity (Figure 3, lane 2). The same two bands appeared at comparable intensities in fraction 6 (Figure 3, lane 3). After incubation of fraction 5 with PCs, the upper band (A1) was much weaker than the lower band (B1) (Figure 3, lane 4). The protein of band A1 had a molecular weight of 66 kDa and was identified as serpin ( $\alpha$ -1-antitrypsin) with an intensity coverage of 42.8% by MS.

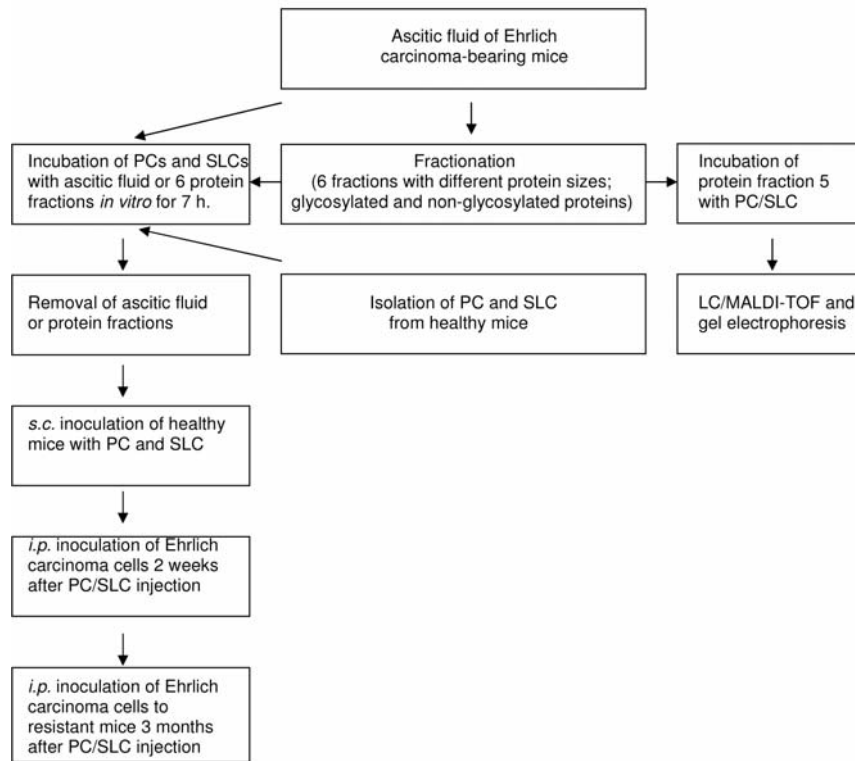


Figure 1. Diagram of the experimental design.

As shown in Figure 3 (lane 4), a weak band appeared at 37.5 kDa. Lane 4 was loaded with 10  $\mu\text{g}$  total protein. When the loading volume was increased to 30  $\mu\text{g}$  total protein, this band was clearly visible (Figure 3, lane 5). As determined by mass spectrometry, this band represents cathepsin L1, with 93.9% intensity coverage (Figure 4).

## Discussion

Recently, we have shown that proteins of ascitic liquids and serum of blood of mice with Ehrlich ascitic carcinoma form complexes (13). Protein complexes always have greater molecular weights than their free subunits. Therefore, tumor-specific activity of fractions 1 and 2 may be explained by the presence of impurities of non-complexed proteins from fraction 3 and by the presence of protein complexes from fraction 3. The biological activities of fractions 1 and 2 were weak and indicate that they were lost during purification. The tumor-specific activity in fraction 5 attests to the participation of glycosylated proteins, which bind to concavalin A by fructose molecules. By contrast, an absence of activity counters there being a role of glycosylated protein binding to concavalin A by mannose molecules. Therefore, it was interesting to compare the biological activity of glycosylated proteins of fractions 5 and 6. Proteins of a molecular size  $>100$  kDa and  $<50$  kDa

were excluded from fractions 5 and 6. Taking all these considerations into account, we investigated proteins of fractions 5 and 6 with a molecular size of  $<100$  kDa and  $>50$  kDa. We found maximal biological activity in fraction 5, whereas fraction 6 was inactive. Interestingly, fraction 6 contained proteins of the same molecular weight, but these proteins were differently glycosylated (more mannose-binding sites) than proteins of fraction 5 (more fructose-binding sites).

The proteomic analyses finally led to the identification of serpin ( $\alpha$ -1-antitrypsin) as candidate protein to explain the biological activity of fraction 5. There is a divergence in the literature concerning the molecular weight of serpin (45 kDa) and its electrophoretic mobility. On the one hand, there are publications reporting a coincidence of electrophoretic mobility of serpin and its real molecular weight of 45 kDa (16). On the other hand, an electrophoretic mobility of approximately 65 kDa has also been described for serpin (17, 18). This significant change in electrophoretic mobility was assumed to be associated with changes in the spatial structure of the protein, or with an interaction of serpin with other proteins, *e.g.* albumin (17, 18). Therefore, the results presented in the current investigation showing the disappearance of a serpin band at 66 kDa are conceivable with the data in the literature.

We identified 40 different proteins, but only one protein of this fraction, serpin, disappeared in our experimental setting.

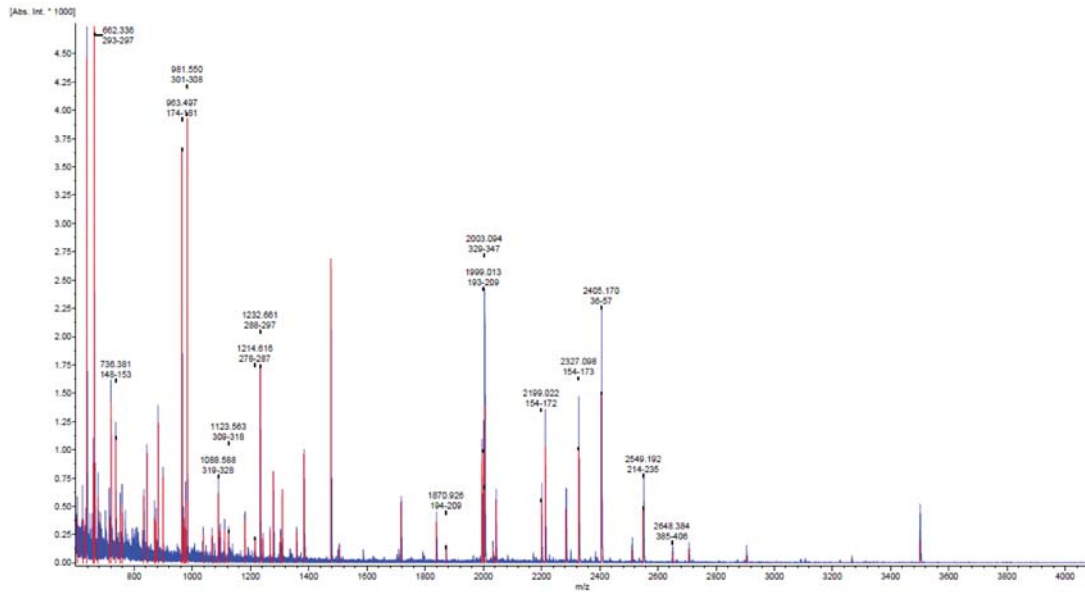


Figure 2. Mass spectrum analysis of serpin ( $\alpha$ -1-antitrypsin).

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 (19, 20). It can, therefore, be speculated that the purging of serpin ( $\alpha$ -1-antitrypsin) in our investigation might lead to a re-expression of cathepsin L1, which is otherwise repressed in the presence of serpin ( $\alpha$ -1-antitrypsin).

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 (21)), 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 (22). 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 (23, 24). Furthermore, serpins may protect parasites against the immune systems of the host (25). Taken together, it can be hypothesized that serpin represents a tissue- and tumor-specific anti-proteinase.

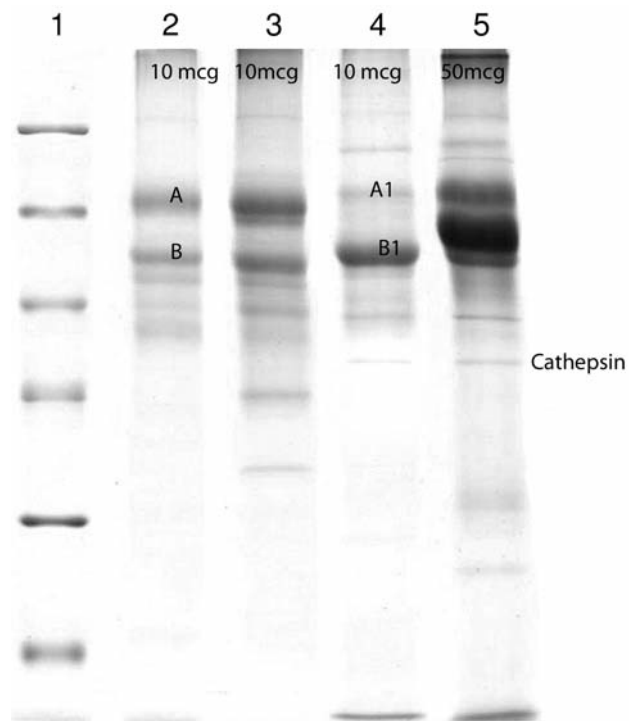


Figure 3. Gel electrophoresis of protein fractions 5 and 6 from Ehrlich carcinoma-bearing mice before and after incubation with peritoneal cells from healthy mice. Lane 1, molecular weight markers (18, 25, 35, 45, 66, and 116 kDa). Lane 2, protein fraction 5 without incubation of peritoneal cells (10  $\mu$ g proteins in lane). Lane 3, protein fraction 6 without incubation of peritoneal cells (10  $\mu$ g proteins on lane). Lanes 4 and 5, protein fraction 5 after incubation with peritoneal cells for 7 h (10  $\mu$ g proteins in lane 4, 50  $\mu$ g proteins in lane 5).



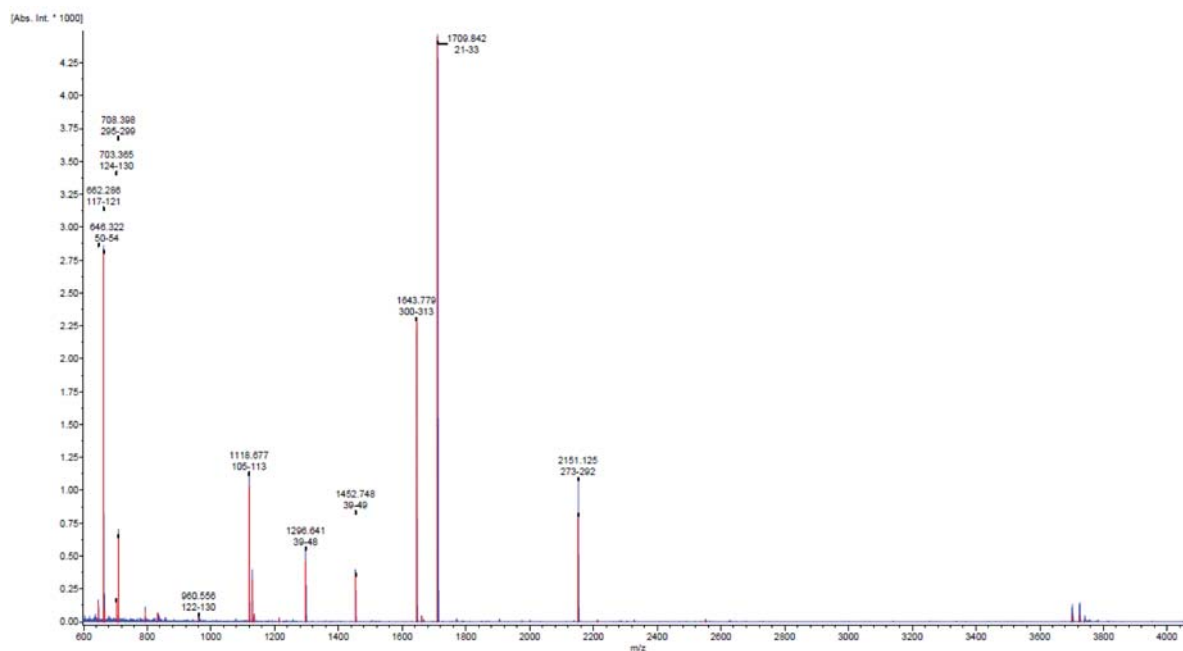


Figure 4. Mass spectrum analysis of cathepsin L1.

The specificity of anti-proteinase activity is frequently determined by glycosylation (26, 27). Different glycosylation patterns of serpin 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 proteases 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 (28). The phenomenon of regulation of different metabolic processes by specific proteases and anti-proteases 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* (29). Surprisingly, this protease did not destroy secreted protein 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 (30). 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 corresponding healthy tissue or in the tumor. Vessel sprouting in other tissues with other tissue-specific proteinases does not take place.

The hypothesis also explains the large variety of different proteinases and anti-proteinases in an organism and the extreme structural complexity of these molecules. This hypothesis might also explain why it has not yet been possible to identify tumor-specific factors in blood serum. In the past, the conceptual idea was built on one or several growth-stimulatory factors assuming their absorption by tumor cells. Here, we assume that specific glycosylation

patterns of serpins (anti-proteinases) might lead to the development of tumors and metastases in those tissues which are normally protected by anti-proteinases. On the other hand, insufficient formation of such anti-proteinases might be accompanied by the development of autoimmune diseases in those tissues which are not protected by serpins. Future investigations will deepen our understanding of how anti-proteinases can transfer specific information to intact cells.

In conclusion, the results of the present investigation show that tumor growth is not solely controlled by a balance of cell division and cell death. Tumor growth represents a much more complex process, which includes serum proteins regulating the activity of immune cells. Therefore, understanding the underlying mechanisms may enable the development of novel treatment strategies against tumors.

## References

- 1 Peeters CF, de Waal RM, Wobbles T, Westphal JR and Ruers TJ: Outgrowth of human liver metastases after resection of the primary colorectal tumor: a shift in the balance between apoptosis and proliferation *Int J Cancer* 119: 1249-1253, 2006.
- 2 Fisher B, Gunduz N, Coyle J, Rudock C and Saffer E: Presence of a growth-stimulating factor in serum following primary tumor removal in mice. *Cancer Res* 49: 1996-2001, 1989.
- 3 Sakon M, Monden M, Gotoh M, Kanai T, Umeshita K, Mori T, Tsubouchi H and Daikuhara Y: Hepatocyte growth factor concentrations after liver resection. *Lancet* 339: 818, 1992.
- 4 Peeters CFJM, Westphal JR, de Waal RMW, Ruiter DJ, Wobbles T and Ruers TJM: Vascular density in colorectal liver metastases increases after removal of the primary tumor in human cancer patients *Int J Cancer* 112: 554-559, 2004.
- 5 Gunduz N, Fisher B and Saffer EA: Effect of surgical removal on the growth and kinetics of residual tumor. *Cancer Res* 39: 3861-3865, 1979.
- 6 Fisher B, Saffer EA and Deutsch M: Influence of irradiation of a primary tumor on the labeling index and estrogen receptor index in a distant tumor focus. *Int J Radiat Oncol Biol Phys* 12: 879-885, 1986.
- 7 Ginat DT, Bhatt S and Dogra VS: Replacement lipomatosis of the kidney: sonographic features. *J Ultrasound Med* 27: 1393-1395, 2008.
- 8 Puttarajappa C and Dhoble A: Mediastinal lipomatosis as a cause of low voltage complexes on electrocardiogram and widened mediastinum: A case report. *Cases J* 1: 171, 2008.
- 9 Jowett C, Mitra P, O'Donnell P and Singh DS: Synovial lipomatosis of hind foot tendon sheaths: case reports and literature review. *Foot Ankle Int* 29: 752-755, 2008.
- 10 Pandzic Jaksic V and Bozkov V: From ancient enigmas to novel paradigms: a depiction of multiple symmetric lipomatosis. *Coll Antropol* 32: 637-640, 2008.
- 11 Goshtasby P, Brooks G and Fielding LP: Lipomatous disorder of the peri-trochanteric soft tissue: case report and review. *Curr Surg* 63: 338-344, 2006.
- 12 Donenko FV, Sitdikova SM, Syrtsev AV, Gradyushko AT, Kiselevskii MV, Serebryakova MV and Efferth T: Hemoglobin-associated proteins isolated from blood serum of Ehrlich carcinoma-bearing mice. *Int J Oncol* 32: 885-893, 2008.
- 13 Donenko FV, Ziganshin RK, Sitdikova SM, Amandzholov BS, Kiselevskii MV and Efferth T: Induction of resistance towards murine tumor development is associated with alterations in glycosylation of blood serum proteins. *Mol Med Rep* 2: 487-495, 2009.
- 14 Goldin A, Kline I and Sofina ZP (eds.): *Experimental Evaluation of Antitumor Drugs in the USA and USSR and Clinical Correlations*. National Cancer Institute Monograph 55. NIH Publication no. 1933. U.S. Department of Health and Human Services. National Institutes of Health, NCI, Bethesda, Maryland, 1980.
- 15 Laemmli UK: Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* 227: 680-685, 1970.
- 16 Roll DE and Glew RH: Isolation and characterization of rat  $\alpha$ -1-antitrypsin. *J Biol Chem* 256: 8190-8196, 1981.
- 17 Finotti P and Pagetta A: Albumin contamination of purified human  $\alpha$ -1-antitrypsin preparation does not affect either structural conformation or the electrophoretic mobility of the inhibitor. *Clin Chim Acta* 264: 133-148, 1997.
- 18 Gupta KV and Gowda LR: Alpha-1 proteinase inhibitor is a heparin binding serpin: molecular interactions with Lys rich cluster of helix-F domain. *Biochimie* 90: 749-761, 2008.
- 19 Takeda A, Yamamoto T, Nakamura Y, Takahashi T and Hibino T: Squamous cell carcinoma antigen is a potent inhibitor of cysteine proteinase cathepsin L. *FEBS Lett* 359: 78-80, 1995.
- 20 Kato H: Expression and function of squamous cell carcinoma antigen. *Anticancer Res* 16: 2149-2153, 1996.
- 21 *Biochemistry*, Stanford University, Second Edition W.H. Freeman and Company, San Francisco, 1980.
- 22 Mistry R, Snashall PD, Totty N, Guz A and Tetley TD: Isolation and characterization of sheep  $\alpha$ -proteinase inhibitor. *Biochem J* 273: 685-690, 1991.
- 23 Kummer JA, Strik MCM, Bladergroen BA and Hack CE: Production, characterization, and use of serpin antibodies. *Methods* 32: 141-149, 2004.
- 24 Law RHP, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI and Whisstock JC: An overview of the serpin superfamily. *Genome Biology* 7: 1-11, 2006.
- 25 Yan Y, Liu S, Song G, Xu Y and Dissous C: Characterization of novel vaccine candidate and serine proteinase inhibitor from *Schistosoma japonicum* (Sj serpin). *Vet Parasitol* 131: 53-60, 2005.
- 26 Lonberg-Holm K, Reed DL, Roberts RC, Hebert RR, Hillman MC and Kutney RM: Three high molecular weight protease inhibitors of rat plasma. *J Biol Chem* 262: 438-445, 1987.
- 27 Silverman GA and Lomas DA: Serpin identification, production, and characterization. *Methods* 32: 71-72, 2004.
- 28 Miller HRP and Pemberton AD: Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 105: 375-390, 2002.
- 29 Trishin AV, Zhdanovich MIu, Savvateeva LV, Toptygin AIu, Donenko FV, Kiselevskii MV, Kurbatova EA, Gruber IM, Elkina SI and Kalina NG: Protease activity of *Klebsiella pneumoniae* of different virulence. *Zh Mikrobiol Epidemiol Immunobiol* 4: 7-11, 2004.
- 30 Gorelik E: Concomitant tumor immunity and the resistance to a second tumor challenge. *Adv Cancer Res* 39: 71-120, 1983.

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