Conformational changes in inter-α-trypsin inhibitor heavy chain 4 activate its tumor-specific activity in mice with B16 melanoma

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Abstract. It is known that blood serum proteins of tumor-bearing mice display tumor-specific activity. However, to date, the nature of this activity has remained elusive, and no tumor-specific proteins have been detected in the blood serum of tumor-bearing animals compared with those in healthy animals. The present study postulated and investigated the hypothesis that the observed tumor-specific activity of the blood serum proteins is not associated with the appearance of novel serum proteins but with changes in the conformation of the existing ones. The present study showed conformational changes of two serum albumin proteins and inter-α-trypsin inhibitor heavy chain 4 (ITIH4) in mice with B16 melanoma compared to tumor-free mice, as determined by differences in the products of proteolysis by proteomic analysis following column chromatography. The differences in the conformation of serum albumin in mice with B16 melanoma and tumor-free mice were accompanied by a change in the interaction of these molecules with the fatty acid spin probe 16-doxyl stearic acid. The differential conformation of ITIH4 in mice with B16 melanoma and that in tumor-free mice was accompanied by inhibition of tumor growth and increased life span. Analysis of the role of protease-anti-proteases (serpins) in the serum of tumor-bearing animals in tumor growth confirmed the hypothesis that tumor growth in the body is mediated, at least in part, via balancing of serpins.

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Key words: tumor growth, serpin, melanoma B16

Introduction

A previous study by our group demonstrated changes in the glycosylation of serum proteins in tumor-bearing mice (1). However, it was not possible to establish a link between the various N-linked oligosaccharide residues and the tumors of animals or cancer patients (1-3). Another previous study by our group showed that the serpin α-anti-trypsin from blood serum inhibits or blocks the growth of tumors (4). The question that arises from these studies is how proteins in the blood serum of tumor-bearing mice as well as in the blood serum of tumor-free mice acquire tumor-associated biological activity. It is known that the blood serum of tumor-bearing animals contains a certain factor which specifically accelerates tumor growth (2,5). However, all attempts to identify this factor as a novel protein in the blood serum of tumor-bearing animals were unsuccessful (5,6). Significant progress has been made toward comprehensive protein expression profiling, and numerous biomarker candidates have been identified; however, none of the reported biomarkers have been proven to be beneficial for patients with cancer (7). It is therefore necessary to identify these factors through their biological activity *in vivo*. The above-mentioned N-linked oligosaccharide residues are part of protein molecules. Changes in these residues [in the previous study by our group they became shorter (4)] are likely to lead to conformational changes of the protein molecule. It is can therefore be assumed that changes in the glycosylation of proteins occur in order to modify the conformation of the protein molecule (8,9). Conformational changes of a protein lead to changes in its biological activity. In organisms displaying tumor growth, no novel proteins were detected in the blood serum compared with those in tumor-free organisms; this may be due to the existing proteins undergoing conformational changes leading to the activation of their tumor-associated activity (10).

The most informative method for studying proteins is proteomic analysis. This analysis involves, in particular, the proteolysis of proteins. It is known that certain proteolytic

enzymes have specific cleavage sites. These sites should fulfill the following requirements: They are required to contain certain amino acids, and furthermore, these sites should be available for their specific cleavage enzymes. If the proteolysis of a protein isolated from the serum of a tumor-bearing mouse and that of the same protein isolated from the serum of a tumor-free mouse results in products, it is indicated that this protein has a differential conformation between the tumor-bearing and the tumor-free mouse. However, until recently, a complete protein denaturation was performed prior to proteomic analysis for better accessibility of trypsin. After the complete denaturation of the proteins with differential conformation, they are expected to have identical availability for trypsin and the trypsinolysis products of these denatured proteins would be identical, therefore not revealing any information regarding differences in protein conformation. This hypothesis explains why proteomic studies have never reported any tumor-specific changes in blood serum proteins.

Tryptic cleavage under soft conditions is challenging; therefore, to obtain a sufficient amount of peptide ions, it is required to use 'semitrypsin' analysis (11,12). This improves the quality and reliability of the identification of proteins and allows for the identification of the protein structure, including various post-translational modifications.

The aim of the present study was to examine the hypothesis that 1) tumor-specific activity of proteins may be associated with changes in their native conformation, e.g., due to changes in their glycosylation and 2) it is possible to determine changes in the native conformation via the proteolysis products of the protein under soft conditions. Semi-tryptic peptide ions were examined and the biological activity of albumin and inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) in tumor-free C57Bl/6 mice and those with B16 melanoma were assessed. The ITIH4 protein is a member of the serpin superfamily (13).

Materials and methods

Tumor and animal experimentation. B16 melanoma cells were obtained from the bank of tumor strains of the N. N. Blokhin Russian Cancer Research Center (Moscow, Russia). Melanoma cells were transplanted subcutaneously to obtain solid tumors into the right hind limb of C57Bl/6 mice ($1x10^6$ cells/mouse diluted in 200 μl RPMI-1640 medium; PanEcho, Moscow, Russia). The experiments were performed using a total of 250 2- to 3-month-old male C57Bl/6 and F1 (C57Bl/6xCBA/Lac) mice (22-24 g) obtained from Stolbovaya Company (Moscow, Russia). The mice received a standard laboratory diet and tap water *ad libitum* and were kept under a natural light/dark cycle. All experiments were performed in accordance with the National Institutes of Health guidelines (14), the legal regulations for animal experimentation in Russia, and the study was approved by the ethics commiteeb of the Institute of Experimental Diagnosis and Therapy of Tumors of the N. N. Blokhin Russian Cancer Research Center (Moscow, Russia). Blood was extracted according to the protocol described by Fisher *et al* (5).

Inductive effect of serum protein on tumor growth. To determine the effect of blood serum proteins, fractions of serum proteins were administered intraperitoneally to healthy mice (100 μg/mouse in 400 μl 0.9% NaCl solution; PanEcho) two weeks prior to tumor transplantation. Each group included 10 animals.

Serum proteins. Blood serum proteins were collected from normal and from tumor-bearing mice. Serum from B16 tumor-bearing mice was collected 30 days after tumor cell injection. Serum proteins were separated into fractions using ultrafiltration membranes (Millipore, Billerica, MA, USA) under air pressure with a nominal molecular weight limit of 300 kDa [PBMK04310; nominal molecular weight limit (NMWL), 300,000] (fraction 1), 100 kDa (PBHK; NMWL, 100,000) (fraction 2) or 50 kDa (PBQK; NMWL, 50,000) (fraction 3). Fraction-3 proteins were diluted in 0.01 M Tris buffer (pH 7.4, containing 0.01% sodium azide) and transferred onto PD-10 columns (Amersham Biosciences, GE Healthcare, Little Chalfont, UK) (4). Proteins were applied to Sepharose Q FF and Sepharose Blue FF columns (XK 16/20; GE Healthcare). Non-bound serum proteins were eluted with 0.01 M Tris buffer (pH 7.4, containing 0.01% sodium azide; Sigma-Aldrich, St Louis, MO, USA), while the bound proteins were eluted using a sodium chloride gradient (0.5 M, 0.01 M Tris buffer, pH 7.4, with 0.01% sodium azide; Sigma-Aldrich) (10) with the help of a GP-250 programmed gradient pump (Pharmacia Biotech, GE Healthcare). Protein elution was monitored in a flow cell (SN 20257; 2 mm; GE Healthcare) at λ=280 nm. The samples containing albumin and serpins were collected and analyzed using an HP 8452A diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) measuring the absorption value at λ=280 nm. Subsequently, proteins were intraperitoneally injected at 100 μg/mouse in 400 μl 0.9% NaCl solution into healthy mice. After 14 days, $1x10^6$ melanoma B16 tumor cells were transplanted into these mice subcutaneously. Each group included 10 animals.

Protein gel electrophoresis. Serpin samples were separated by 12.5% SDS-PAGE and stained with Coomassie blue (Sigma-Aldrich) according to Laemmli (15). Samples subjected to SDS-PAGE were solubilized in a sample buffer (Sigma-Aldrich) containing 63 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 30 M bromophenol blue (Sigma-Aldrich). 2-Mercaptoethanol 5% (v/v) (Sigma-Aldrich) was conditionally added or omitted in the sample buffer. 12.5% acrylamide gels with a bisacrylamide/acrylamide ratio of 0.8:30 were used. Samples were applied in quantities of 10 and 50 μg protein/lane to evaluate all components of the protein complexes. Protein gel electrophoresis was used for determination of the mass of ITIH4 with tumor specific activity.

Assessment of albumin binding using electron spin resonance (ESR). ESR spectra were measured for each sample using a commercially available ESR spectrometer (AXM-09; ESR-Analyzer/MMS; MedInnovation GmbH, Berlin, Germany). The principle of this technique is the measurement of albumin binding variables, achieved by a fatty acid spin probe. The binding variables of the spin probe were determined at different permutations of the ethanol concentration as well as the ratio of spin probe and albumin concentration. Variation of the ethanol concentration allowed for the assessment of binding variables of the spin probe to albumin under different hydrophobic condi-

Table I. Semi-tryptic peptides of albumin identified in tumor-bearing mice and tumor-free mice.

B16 melanoma (m/z of peptide ion)	Tumor-free mice (m/z of peptide ion)	Amino acids (start-end)
777.3831	777.4126	199-205
1149.6602	1149.6265	66-75
1250.6354	1250.5844	35-44
1439.8602	1439.7895	439-452
1479.9253	1479.8529	45-57
1681.9364	1681.8677	243-257
3870.3227	3869.8416	25-57

Table II. Semi-tryptic peptides of albumin identified in tumor-bearing mice only.

Peptide ion (m/z)	Amino acids (start-end)
960.4622	484-490
1019.6167	234-242
1272.6371	211-221
1902.0049	153-168
2711.6178	35-57
2982.4342	563-588
3369.7339	559-588
342.693	410-438

tions. Changes in the ratio of spin probe to albumin enabled the measurement of the binding affinity of albumin to the spin probe.

Commercial 16-doxyl stearic acid (Sigma-Aldrich) was used as spin probe. This compound was selected due to the exceptionally high binding constant of albumin to stearic acid ($6.9x10^7$ l/mol), which produces >99.9% binding of the spin probe to albumin. Ethanol, extra pure, (Merck Millipore) was used for modifying the binding affinity of the fatty acid spin probe to albumin. The final concentrations of ethanol (mol/l) and spin probe (10^{-3} mol/l) were 2.9 and 0.83 in aliquot 1, 3.4 and 1.61 in aliquot 2, and 3.8 and 2.34 in aliquot 3, respectively (16).

Mass spectrometry. For mass spectrometric analysis, samples of protein were only used after purification using Sephadex Q FF columns. Proteins were diluted in 50 mM ammonium bicarbonate solution and transferred onto PD-10 columns (W359685; GE Healthcare). The final concentration of the proteins in the samples was 1 mg/ml. Solely following column chromatography without electrophoresis, the samples were incubated at 37˚C in 100 μl 20 mM ammonium bicarbonate containing 5 ng/μl trypsin, chymotrypsin and 10% acetonitrile overnight (all from Sigma-Aldrich). Peptides were concentrated using SEP-PAK C18 cartridges (Millipore).

Mass spectra were recorded using an Ultraflex Extreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a neodymium laser. To analyze mass spectra, FlexAnalysis software, version 3.3 (Bruker Daltonics, Bremen, Germany) was used. Aliquots of the samples were mixed on a steel target with a solution of 2,5-dihydroxybenzoic acid (20 mg/ml) in 30% (v/v) acetonitrile (Sigma-Aldrich) with 0.5% (v/v) trifluoroacetic acid. The $[MH]^+$ molecular ions were analyzed in linear (proteins) or reflector (peptides) mode, and the m/z ratios were accurate to 30 ppm. Fragment ion spectra were obtained in Lift mode with a mass accuracy of better than 1 Da. Proteases cleaved peptides were used in the peptide mixtures. The data were processed using Flex Analysis 2.2 software (Bruker Daltonics), and peaks of trypsin fragments contained in samples were used for calibration. The following search parameters were used: Accuracy of mass determination = 100 ppm, NCBInr database, Rodentia taxon (rodent), one missed cleavage and possible methionine oxidation. The proteins were identified using the Mascot version 2.2.07 search software (peptide fingerprint option; www.matrixscience.com). The search was conducted in the NCBI databases and/or EST vertebrates. For the search of candidate proteins in combined ms + (ms-ms) data, Biotools, version 3.0 (Bruker Daltonics) was used. If the value of the Score parameter calculated for each protein exceeded 70, identification was accepted as reliable.

Statistical analysis. Statistical evaluation was performed using Fisher's exact test or Student's t-test. Data are presented as the mean ± standard deviation and Microsoft Office Excel (14.0.6112.5000) software was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Tumor-free mice and mice with B16 melanoma have differential proteolytic fragments of serum albumin. By means of column chromatography, a fraction corresponding to albumin was extracted from the blood serum of tumor-free mice and the blood serum of mice with B16 melanoma. Subsequent proteomic analysis identified the serum precursor of albumin in the two cases (Fig. 1A and B). Thus, albumin extracted from mice with the tumor has the same amino acid sequence as albumin extracted from the tumor-free mice.

In addition, the data presented in Fig. 1A and B show that proteolytic analysis of the albumin from tumor-free mice identified a greater number of semi-tryptic peptide ions compared with that in mice with B16 melanoma (64 vs 15 peptides, respectively). Normal mice and those with B16 melanoma had seven peptides

A

gi|163310765 **Mass:** 68648 **Score:** 83 **Expect:** 0.0055 **Matches:** 64
serum albumin precursor [Mus musculus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
761.3758	760.3685	760.3967	-36.97	563	569	0	---	K.ATAEQLK.T + Deamidated (NQ)
774.3856	773.3783	773.4072	-37.31	89	95	0	---	K.SLHTLFG.D
777.4126	776.4053	776.3738	40.6	199	205	0	---	K.ESCLTPK.L
808.4006	807.3933	807.3650	35.0	243	249	0	---	R.LSQTFPN.A + 2 Deamidated (NQ)
828.4451	827.4379	827.4752	-45.16	407	413	1	---	E.EPKNLVK.T + Deamidated (NQ)
830.3876	829.3803	829.3970	-20.10	355	360	0	---	F.LYEYSR.R
873.4370	872.4297	872.4141	17.9	361	367	1	---	R.RHPDYSV.S
894.4789	893.4716	893.4494	24.9	250	257	0	---	N.ADFAEITK.L
918.4799	917.4726	917.4528	21.6	536	543	0	---	S.DICTLPEK.E
940.4717	939.4644	939.4888	-25.99	1	7	1	---	-.MKWVTFL.L + Oxidation (M)
962.5168	961.5095	961.5233	-14.28	427	434	0	---	G.FQNAILVR.Y + 2 Deamidated (NQ)
984.4970	983.4897	983.5076	-18.24	50	57	0	---	I.AFSQYLQK.C
989.4819	988.4747	988.4726	2.07	29	36	1	---	K.SEIAHRYN.D
1019.6093	1018.6021	1018.6215	-19.11	2	9	1	---	M.KWVTFLLL.L
1038.4667	1037.4594	1037.4708	-10.99	302	309	0	---	T.CCDKPLLK.K + Cysteinyl (C)
1062.4408	1061.4335	1061.4014	30.3	222	229	1	---	R.MKCSSMQK.F + Deamidated (NQ); Cysteinyl (C)
1132.5209	1131.5136	1131.4576	49.5	143	152	0	---	P.EAEAMCTSFK.E + Oxidation (M)
1149.6265	1148.6192	1148.6077	9.96	66	75	0	---	K.LVQEVTDFAK.T
1179.5957	1178.5884	1178.5675	17.8	537	545	1	---	D.ICTLPEKEK.Q + Cysteinyl (C)
1250.5844	1249.5771	1249.5727	3.52	35	44	0	---	R.YNDLGEQHFK.G
1282.5380	1281.5307	1281.5547	-18.74	331	341	0	---	A.ADFVEDQEVCK.N
1292.6641	1291.6568	1291.6349	16.9	169	178	1	48	R.RHPYFYAPEL.L
1346.5172	1345.5100	1345.5166	-4.92	106	117	0	---	R.ENYGELADCCTK.Q + Deamidated (NQ)
1406.7102	1405.7029	1405.6738	20.7	34	44	1	---	H.RYNDLGEQHFK.G
1439.7895	1438.7823	1438.7780	2.98	439	452	0	---	K.APQVSTPTLVEAAR.N
1479.8529	1478.8456	1478.8497	-2.74	45	57	0	---	K.GLVLIAFSQYLQK.C
1501.8569	1500.8497	1500.8399	6.54	544	556	3	---	K.EKQIKKQTALAEL.V + 2 Deamidated (NQ)
1530.6545	1529.6472	1529.6569	-6.29	118	129	1	---	K.QEPERNECFLQH.K + Deamidated (NQ)
1552.6675	1551.6602	1551.6044	36.0	572	584	0	---	V.MDDFAQFLDTCCK.A + Oxidation (M)
1626.7882	1625.7809	1625.7243	34.8	334	347	1	---	F.VEDQEVCKNYAEAK.D + Deamidated (NQ)
1681.8677	1680.8605	1680.8359	14.6	243	257	0	---	R.LSQTFPNADFAEITK.L
1703.8686	1702.8613	1702.8566	2.78	425	438	1	---	E.YGFQNAILVRYTQK.A + 3 Deamidated (NQ)
1725.8446	1724.8373	1724.8342	1.81	24	37	3	---	R.REAHKSEIAHRYND.L
1840.8889	1839.8817	1839.9117	-16.33	211	225	4	---	K.EKALVSSVRQRMKCS.S + Cysteinyl (C)
1888.8641	1887.8568	1887.8593	-1.34	282	298	1	---	R.AELAKYMCENQATISSK.L + 2 Deamidated (NQ)
1901.9604	1900.9531	1900.9570	-2.05	419	434	1	---	L.YEKLGEYGFQNAILVR.Y + 2 Deamidated (NQ)
1918.8436	1917.8363	1917.8567	-10.60	50	65	1	---	I.AFSQYLQKCSYDEHAK.L + Deamidated (NQ)
1973.9300	1972.9227	1972.9598	-18.80	258	275	2	---	K.LATDLTKVNKECCHGDLL.E + Deamidated (NQ)
2123.9409	2122.9336	2122.9380	-2.06	218	233	4	---	S.VRQRMKCSSMQKFGER.A + 2 Deamidated (NQ); 2 Oxidation (M); Cysteinyl (C)
2267.1002	2266.0929	2266.0398	23.4	133	152	0	---	D.NPSLPPFERPEAEAMCTSFK.E + Oxidation (M)
2285.0967	2284.0894	2284.0827	2.93	584	604	3	---	C.KAADKDTCFSTEGPNLVTRCK.D + Deamidated (NQ)
2302.0979	2301.0907	2301.1351	-19.31	389	409	0	---	A.NPPACYGTVLAEFQPLVEEPK.N
2383.1272	2382.1200	2382.1063	5.75	123	142	1	---	R.NECFLQHKDDNPSLPPFERP.E
2767.3422	2766.3349	2766.3278	2.57	457	481	2	---	R.VGTKCCTLPEDQRLPCVEDYLSAIL.N + Deamidated (NQ)
2829.3899	2828.3826	2828.2601	43.3	453	476	3	9	R.NLGRVGTKCCTLPEDQRLPCVEDY.L + Deamidated (NQ); Cysteinyl (C)
2846.3625	2845.3552	2845.3997	-15.63	445	469	3	---	T.PTLVEAARNLGRVGTKCCTLPEDQR.L + Cysteinyl (C)
2857.4300	2856.4228	2856.3859	12.9	373	399	3	---	R.LAKKYEATLEKCCAEANPPACYGTVLA.E
2947.4286	2946.4213	2946.4210	0.09	284	309	2	---	E.LAKYMCENQATISSKLQTCCDKPLLK.K + 2 Deamidated (NQ); Oxidation (M)
2982.5004	2981.4931	2981.5208	-9.28	45	70	2	---	K.GLVLIAFSQYLQKCSYDEHAKLVQEV.T + Deamidated (NQ)
3086.5296	3085.5223	3085.4525	22.6	414	438	2	---	K.TNCDLYEKLGEYGFQNAILVRYTQK.A + Deamidated (NQ); Cysteinyl (C)
3110.6117	3109.6044	3109.5471	18.4	435	462	4	---	R.YTQKAPQVSTPTLVEAARNLGRVGTKCC.T + Cysteinyl (C)
3232.5635	3231.5563	3231.5614	-1.58	560	588	3	---	H.KPKATAEQLKTVMDDFAQFLDTCCKAADK.D + Oxidation (M)
3241.6137	3240.6064	3240.6231	-5.14	440	469	3	---	A.PQVSTPTLVEAARNLGRVGTKCCTLPEDQR.L + 2 Deamidated (NQ)
3249.5743	3248.5670	3248.4233	44.3	183	210	2	10	A.EQYNEILTQCCAEADKESCLTPKLDGVK.E + 2 Deamidated (NQ); Cysteinyl (C)
3267.5911	3266.5838	3266.5329	15.6	414	441	3	---	K.TNCDLYEKLGEYGFQNAILVRYTQKAPQ.V + 5 Deamidated (NQ)
3369.6769	3368.6696	3368.6387	9.19	37	65	2	---	N.DLGEQHFKGLVLIAFSQYLQKCSYDEHAK.L + 2 Deamidated (NQ)
3420.6079	3419.6006	3419.6379	-10.90	281	309	3	---	D.RAELAKYMCENQATISSKLQTCCDKPLLK.K + Oxidation (M); Cysteinyl (C)
3438.5452	3437.5379	3437.4814	16.5	461	487	2	---	K.CCTLPEDQRLPCVEDYLSAILNRVCLL.H + 2 Deamidated (NQ); 3 Cysteinyl (C)
3552.5752	3551.5679	3551.5135	15.3	94	122	3	---	L.FGDKLCAIPNLRENYGELADCCTKQEPER.N + 2 Deamidated (NQ); 2 Cysteinyl (C)
3869.8416	3868.8343	3869.0012	-43.14	25	57	3	48	R.EAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQK.C
3985.8719	3984.8646	3984.8617	0.74	307	341	2	---	P.LLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCK.N + Deamidated (NQ); Cysteinyl (C)
4082.4150	4081.4077	4081.5726	-40.40	106	137	3	---	R.ENYGELADCCTKQEPERNECFLQHKDDNPSLP.P + 3 Deamidated (NQ); 3 Cysteinyl (C)
4204.5986	4203.5913	4203.7377	-34.82	106	139	3	---	R.ENYGELADCCTKQEPERNECFLQHKDDNPSLPPF.E + 2 Cysteinyl (C)
6344.6375	6343.6302	6343.6570	-4.23	106	160	4	---	R.ENYGELADCCTKQEPERNECFLQHKDDNPSLPPFERPEAEAMCTSFKENPTTFMG.H + 5 Deamidated (NQ); Oxidation (M)

B

gi|163310765 **Mass:** 68648 **Score:** 244 **Expect:** 4.8e-19 **Matches:** 15
serum albumin precursor [Mus musculus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
777.3831	776.3758	776.3738	2.59	199	205	0	---	K.ESCLTPK.L
960.4622	959.4549	959.4568	-2.02	484	490	0	---	R.VCLLHEK.T + Cysteinyl (C)
1019.6167	1018.6094	1018.5712	37.5	234	242	1	---	R.AFKAWAVAR.L
1149.6602	1148.6529	1148.6077	39.3	66	75	0	---	K.LVQEVTDFAK.T
1250.6354	1249.6282	1249.5727	44.3	35	44	0	---	R.YNDLGEQHFK.G
1272.6371	1271.6298	1271.7310	-79.54	211	221	2	---	K.EKALVSSVRQR.M
1439.8602	1438.8529	1438.7780	52.1	439	452	0	---	K.APQVSTPTLVEAAR.N
1479.9253	1478.9180	1478.8497	46.2	45	57	0	---	K.GLVLIAFSQYLQK.C
1681.9364	1680.9291	1680.8359	55.5	243	257	0	---	R.LSQTFPNADFAEITK.L
1902.0049	1900.9976	1900.8890	57.1	153	168	0	---	K.ENPTTFMGHYLHEVAR.R
2711.6178	2710.6105	2710.4119	73.3	35	57	1	164	R.YNDLGEQHFKGLVLIAFSQYLQK.C
2982.4342	2981.4270	2981.3279	33.2	563	588	2	---	K.ATAEQLKTVMDDFAQFLDTCCKAADK.D + Cysteinyl (C)
3369.7339	3368.7267	3368.6203	31.6	559	588	3	---	K.HKPKATAEQLKTVMDDFAQFLDTCCKAADK.D + Oxidation (M)
3420.6930	3419.6857	3419.7547	-20.18	410	438	3	---	K.NLVKTNCDLYEKLGEYGFQNAILVRYTQK.A
3870.3227	3869.3154	3869.0012	81.2	25	57	3	52	R.EAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQK.C

Figure 1. Summary report of the identification of serum albumin precursor and semi-tryptic peptides (A) for tumor-free mice and (B) for mice with B16 melanoma. N, arginine; Q, glutamine; C, cysteine; M, methionine.

in common, and eight peptides were found exclusively in mice with B16 melanoma (Tables I and II). However, a number of the peptides which identified in mice with B16 melanoma partially or completely overlapped with a significant number of the peptides identified in tumor-free mice. This resulted in a larger number of identified peptides in tumor-free mice compared with that in mice with B16 melanoma (Table III).

In tumor-free mice, seven peptides were identified to partially overlap with those identified in mice with B16 melanoma with regard to their amino acid sequences at 35-57 and the m/z ratio of 2,711.6178, and two peptides were identified to partially overlap with regard to their amino acid sequences at 211-221 and the m/z ratio of 1,272.6371. Furthermore, in tumor-free mice, two peptides were identified to partially overlap with those found in mice with B16 melanoma with regard to their amino acid sequences at 243-257 and the m/z ratio of 1,681.9364. In tumor-free mice, six peptides were identified to partially or completely overlap with those from

Table III. Overlapping peptides of albumin in tumor-bearing mice and in tumor-free mice.

Animals	Peptide ion (m/z)	Start-end	Amino acid sequence
B16 melanoma	2711.6178	35-57	R.YNDLGEQHFKGLVLIAFSQYLQK.C
Tumor-free mice	1725.8446	24-37	R.REAHKSEIAHRYND.L
Tumor-free mice	989.4819	29-36	K.SEIAHRYN.D
Tumor-free mice	1406.7102	34-44	H.RYNDLGEQHFK.G
Tumor-free mice	3369.6769	37-65	N.DLGEQHFKGLVLIAFSQYLQKCSYDEHAK.L +2 deamidated (NQ)
Tumor-free mice	2982.5004	45-70	K.GLVLIAFSQYLQKCSYDEHAKLVQEV.T + deamidated (NQ)
Tumor-free mice	984.497	50-57	I.AFSQYLQK.C
Tumor-free mice	1918.8436	50-65	I.AFSQYLQKCSYDEHAK.L + deamidated (NQ)
B16 melanoma	1272.6371	211-221	K.EKALVSSVRQR.M
Tumor-free mice	1840.8889	211-225	K.EKALVSSVRQRMKCS.S + cysteinyl (C)
Tumor-free mice	2123.9409	218-233	S.VRQRMKCSSMQKFGER.A + 2 deamidated (NQ)
Tumor-free mice	1681.8677	243-257	R.LSQTFPNADFAEITK.L
B16 melanoma	1681.9364	243-257	R.LSQTFPNADFAEITK.L
Tumor-free mice	808.4006	243-249	R.LSQTFPN.A + 2 deamidated (NQ)
Tumor-free mice	894.4789	250-257	N.ADFAEITK.L
B16 melanoma	3420.693	410-438	K.NLVKTNCDLYEKLGEYGFQNAILVRYTQK.A
Tumor-free mice	3086.5296	414-438	K.TNCDLYEKLGEYGFQNAILVRYTQK.A + Deamidated (NQ)
Tumor-free mice	3267.5911	414-441	K.TNCDLYEKLGEYGFQNAILVRYTQKAPQ.V +5 deamidated (NQ)
Tumor-free mice	1901.9604	419-434	L.YEKLGEYGFQNAILVR.Y + 2 deamidated (NQ)
Tumor-free mice	1703.8686	425-438	E.YGFQNAILVRYTQK.A + 3 deamidated (NQ)
Tumor-free mice	962.5168	427-434	G.FQNAILVR.Y + 2 deamidated (NQ)
Tumor-free mice	3110.6117	435-462	R.YTQKAPQVSTPTLVEAARNLGRVGTKCC.T + cysteinyl (C)
Tumor-free mice	1439.7895	439-452	K.APQVSTPTLVEAAR.N
B16 melanoma	1439.8602	439-452	K.APQVSTPTLVEAAR.N
Tumor-free mice	3241.6137	440-469	A.PQVSTPTLVEAARNLGRVGTKCCTLPEDQR.L + 2 deamidated (NQ)
Tumor-free mice	2846.3625	445-469	T.PTLVEAARNLGRVGTKCCTLPEDQR.L + cysteinyl (C)
B16 melanoma	3369.7339	559-588	K.HKPKATAEQLKTVMDDFAQFLDTCCKAADK. D + oxidation (M)
B16 melanoma	2982.4342	563-588	K.ATAEQLKTVMDDFAQFLDTCCKAADK. D + cysteinyl (C)
Tumor-free mice	3232.5635	560-588	H.KPKATAEQLKTVMDDFAQFLDTCCKAADK. D + oxidation (M)
Tumor-free mice	761.3758	563-569	K.ATAEQLK.T + deamidated (NQ)
Tumor-free mice	1552.6675	572-584	V.MDDFAQFLDTCCK.A + oxidation (M)
Tumor-free mice	2285.0967	584-604	C.KAADKDTCFSTEGPNLVTRCK.D + deamidated (NQ)

N, arginine; Q, glutamine; C, cysteine; M, methionine.

mice with B16 melanoma in their amino acid sequences at 410-438 and an m/z 3,420.693. In addition, in tumor-free mice, two peptides were identified to partially overlap with peptides from mice with B16 melanoma in their amino acid sequences at 439-452 and the m/z ratios of 1,439.8602 and 1,439.7895. Finally, in tumor-free mice, four peptides were identified to partially or fully overlap with two peptides in mice with B16 melanoma regarding the amino acid sequences 559-588 with the m/z ratio of 3,369.7339 and 563-588 with the m/z ratio of 2,982.4342. The large number and variation of proteolysis products of the serum proteins from tumor-free mice compared to those from mice with melanoma B16, indicates the mobility of these specific regions in the former and the rigidity of the same parts of the molecule in the latter. The

A

gi|16741341 **Mass:** 104566 **Score:** 68 **Expect:** 0.18 **Matches:** 32

Inter alpha-trypsin inhibitor, heavy chain 4 [Mus musculus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
674.3995	673.3922	673.3759	24.3	98	103	1	---	E.KAEAQK.Q
773.4531	772.4458	772.4517	-7.57	867	873	0	---	R.GLMLLLN.D
793.4153	792.4080	792.4018	7.83	910	916	0	---	K.VQGVDYL.A
808.3843	807.3770	807.4126	-44.10	529	534	1	---	Q.QEKEFK.S
871.3901	870.3828	870.4083	-29.29	497	504	0	---	K.LQDQGPDV.L
881.4796	880.4723	880.4807	-9.45	822	827	2	---	T.EYKWKK.T
997.4688	996.4615	996.4553	6.25	923	931	0	---	K.LSYQEGFPG.A
1016.4871	1015.4798	1015.5008	-20.67	265	273	0	---	A.PENLPTMSK.N
1043.4895	1042.4823	1042.5157	-32.11	77	85	0	---	K.AFITNFSMI.I
1187.6310	1186.6237	1186.6207	2.56	802	811	0	---	P.HLQVHATPER.L
1311.6510	1310.6438	1310.6143	22.5	895	905	0	---	V.WEPPVEPDNTK.R
1405.7547	1404.7474	1404.6748	51.7	171	181	0	---	K.HLQMDIYIFEP.Q
1420.7739	1419.7667	1419.7722	-3.88	716	728	1	---	Y.PADPHLVVTEKSK.E
1457.7543	1456.7470	1456.7714	-16.77	820	830	3	---	K.NTEYKWKKTLF.S
1563.8580	1562.8508	1562.8893	-24.63	812	824	3	---	R.LVVTRGRKNTEYK.W
1626.8520	1625.8447	1625.8058	23.9	1	14	1	14	-.MKSPAPAHMWNLVL.F + 2 Oxidation (M)
1666.8365	1665.8292	1665.8468	-10.55	280	294	3	---	I.DKSGSMSGKKIQQTR.E + Oxidation (M)
1704.8222	1703.8149	1703.9505	-79.58	163	176	1	---	K.VRPQQLVKHLQMDI.Y
1757.8543	1756.8471	1756.8793	-18.35	1	15	1	---	-.MKSPAPAHMWNLVLF.L + Oxidation (M)
1791.8798	1790.8725	1790.9104	-21.15	656	673	1	---	R.ASRQYIPPGFPGPPGPPG.F
1794.9294	1793.9221	1793.9272	-2.82	104	121	2	---	K.QYSAAVGRGESAGIVKTT.G
1812.8909	1811.8836	1811.9451	-33.97	272	288	2	---	M.SKNVIFVIDKSGSMSGK.K + Oxidation (M)
1911.9322	1910.9249	1910.9414	-8.62	786	801	0	---	K.YENTGFSWLEVTIQKP.H
1973.9343	1972.9271	1972.9014	13.0	729	746	0	81	K.ESTIPEESPNPDHPQVPT.I
2156.0816	2155.0743	2155.1572	-38.42	544	560	1	---	F.MERLWALLTIQQQLEQR.I
2189.0602	2188.0529	2188.0284	11.2	727	746	1	---	K.SKESTIPEESPNPDHPQVPT.I
2415.1894	2414.1821	2414.2668	-35.05	140	158	2	---	K.ITFELIYQELLQRRLGMYE.L
2586.3754	2585.3681	2585.3761	-3.09	612	635	2	---	V.GDGMQRLPLAAQAHPFRPPVRGSK.L
2943.3268	2942.3195	2942.4082	-30.13	171	195	0	---	K.HLQMDIYIFEPQGISILETESTFMT.P
2977.4323	2976.4250	2976.3923	11.0	429	454	2	---	K.MALDNGGLARRIYEDSDSALQLQDFY.H + Oxidation (M)
3202.4850	3201.4777	3201.5191	-12.94	404	431	1	---	R.EAINGQYSLFCLGFGFDVNYPFLEKMAL.D + Oxidation (M)
4205.1424	4204.1352	4204.1586	-5.58	188	224	3	6	L.ETESTFMTPELANALTTSQNKTKAHIRFKPTLSQQQK.S

B

gi|16741341 **Mass:** 104566 **Score:** 68 **Expect:** 0.19 **Matches:** 35

Inter alpha-trypsin inhibitor, heavy chain 4 [Mus musculus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
674.3956	673.3883	673.3759	18.5	98	103	1	---	E.KAEAQK.Q
733.3792	732.3719	732.3840	-16.48	490	496	0	---	S.EMVVAGK.L
790.4290	789.4217	789.4055	20.6	267	273	0	---	E.NLPTMSK.N
905.5092	904.5019	904.4726	32.4	40	47	1	---	T.VDSRVSSR.F
916.5098	915.5025	915.5138	-12.28	111	119	1	---	G.RGESAGIVK.T
943.5501	942.5428	942.4771	69.8	859	866	0	---	L.LSLDDPQR.G
1040.5248	1039.5176	1039.4968	20.0	280	289	2	---	I.DKSGSMSGKK.I + Oxidation (M)
1126.5997	1125.5924	1125.5455	41.7	697	705	0	---	D.FSLQPSYER.M
1228.7227	1227.7155	1227.7438	-23.08	295	305	2	---	R.EALVKILKDLS.P
1277.6970	1276.6897	1276.7139	-18.98	906	916	2	---	K.RTVKVQGVDYL.A
1330.7948	1329.7875	1329.8384	-38.28	826	837	2	---	W.KKTLFSVLPGLK.M
1405.7664	1404.7591	1404.6748	60.1	171	181	0	---	K.HLQMDIYIFEP.Q
1457.7798	1456.7725	1456.7714	0.75	820	830	3	---	K.NTEYKWKKTLF.S
1545.8697	1544.8624	1544.8423	13.0	44	57	2	---	R.VSSRFAHTVVTSRV.V
1563.8820	1562.8747	1562.8893	-9.31	812	824	3	---	R.LVVTRGRKNTEYK.W
1609.8461	1608.8388	1608.8471	-5.17	896	909	2	---	W.EPPVEPDNTKRTVK.V
1626.8700	1625.8627	1625.8058	35.0	1	14	1	14	-.MKSPAPAHMWNLVL.F + 2 Oxidation (M)
1681.8909	1680.8836	1680.8446	23.2	535	547	2	---	K.SPKYIFHNFMERL.W
1704.8644	1703.8571	1703.9505	-54.81	163	176	1	---	K.VRPQQLVKHLQMDI.Y
1780.0213	1779.0140	1778.9309	46.7	279	294	3	---	V.IDKSGSMSGKKIQQTR.E + Oxidation (M)
1796.9473	1795.9400	1795.9502	-5.68	272	288	2	---	M.SKNVIFVIDKSGSMSGK.K
1826.9788	1825.9715	1825.9504	11.6	827	842	2	---	K.KTLFSVLPGLKMTMNM.M + Oxidation (M)
1926.9701	1925.9629	1925.9083	28.3	837	853	1	---	L.KMTMNMMGLLQLSGPDK.V + 2 Oxidation (M)
1973.9866	1972.9793	1972.9014	39.5	729	746	0	81	K.ESTIPEESPNPDHPQVPT.I
1987.0943	1986.0871	1986.0040	41.8	659	678	0	---	R.QYIPPGFPGPPGPPGFPAPP.G
2119.0905	2118.0833	2118.1031	-9.36	767	785	1	---	S.EKPMKLFVDPSQGLEVTGK.Y + Oxidation (M)
2156.1239	2155.1167	2155.1572	-18.79	544	560	1	---	F.MERLWALLTIQQQLEQR.I
2226.2278	2225.2205	2225.2168	1.67	547	566	1	---	R.LWALLTIQQQLEQRISASGA.E
2354.1529	2353.1457	2353.0725	31.1	404	424	0	---	R.EAINGQYSLFCLGFGFDVNYP.F
2427.2856	2426.2783	2426.1929	35.2	838	859	1	---	K.MTMNMMGLLQLSGPDKVTIGLL.S + 4 Oxidation (M)
2544.3711	2543.3639	2543.3457	7.13	140	159	2	---	K.ITFELIYQELLQRRLGMYEL.L + Oxidation (M)
2878.4584	2877.4511	2877.2916	55.4	246	271	0	---	R.SDSGGSIQIEEGYFVHHFAPENLPTM.S + Oxidation (M)
2977.5123	2976.5050	2976.5432	-12.85	538	560	1	---	K.YIFHNFMERLWALLTIQQQLEQR.I
3499.9311	3498.9238	3498.9068	4.88	610	641	3	---	V.EVGDGMQRLPLAAQAHPFRPPVRGSKLMTVLK.G
4205.2788	4204.2715	4204.1586	26.9	188	224	3	6	L.ETESTFMTPELANALTTSQNKTKAHIRFKPTLSQQQK.S

Figure 2. Summary report of the identification of the inter-alpha-trypsin inhibitor heavy chain 4 and semi-tryptic peptides (A) for tumor-free mice and (B) for mice with B16 melanoma. M, methionine.

increase in rigidity of the albumin molecule during tumor growth may be due to the formation of cross-links within the molecule. This process may be similar to the transformation of fibrin to form internal cross-links (17). This process may make proteins in animals with tumors more rigid compared to those of tumor-free animals and leads to a decrease in the number of proteolysis products.

Tumor-free mice and mice with B16 melanoma have differential proteolytic fragments of serum ITIH4. Another protein isolated by column chromatography was the protein fraction corresponding to ITIH4. Similarly to the case of albumin, it was necessary to verify that proteins with the same amino acid sequence were analyzed in the protein extracted from the blood serum of the mice with B16 melanoma and that from the blood serum of tumor-free mice. As shown in Fig. 2A and B, proteomic analysis identified ITIH4 in the two groups.

The total quantity of identified peptides following proteolysis of ITIH4 from the serum of tumor-free mice was 32, that of mice with B16 melanoma was 35. Thus, by contrast with albumin, in the case of serpin, no significant difference was observed in the number of identified peptide fragments of ITIH4. As discussed above, the lack of variations in the number of identified peptides in plasma during tumor growth may be explained by various

Table IV. Semi-tryptic peptides of the inter-alpha-trypsin inhibitor heavy chain 4 identified in tumor-bearing and tumor-free mice.

Peptide ion in B16 melanoma (m/z)	Peptide ion in tumor-free mice (m/z)	Amino acids (start-end)
674.3956	674.3995	98-103
1405.7664	1405.7547	171-181
1457.7798	1457.7543	820-830
1563.882	1563.858	812-824
1626.8700	1626.852	1-14
1704.8644	1704.8222	163-176
1973.9866	1973.9343	729-746
2156.1239	2156.0816	544-560
1796.9473	1812.8909	272-288
4205.2788	4205.1424	188-224

Table V. Semi-tryptic peptides of the inter-alpha-trypsin inhibitor heavy chain 4 identified in tumor-bearing mice only.

Peptide ion (m/z)	Amino acids (start to end)
733.3792	490-496
790.4290	267-273
905.5092	40-47
916.5098	111-119
943.5501	859-866
1040.5248	280-289
1126.5997	697-705
1228.7227	295-305
1277.6970	906-916
1330.7948	826-837
1545.8697	44-57
1609.8461	896-909
1780.0213	279-294
1826.9788	827-842
1926.9701	837-853
1987.0943	659-678
2119.0905	767-785
2354.1529	404-424
2427.2856	838-859
2544.3711	140-159
2977.5123	538-560
3499.9311	610-641

mechanisms of modification of the proteins in the body. Serpin, unlike albumin, is a glycosylated protein and its spatial structure changes due to glycosylation, not due to internal cross-links. Glycosylation-induced changes of blood serum proteins during tumor growth are discussed above (2).

Among the identified peptide fragments of ITIH4 following proteolysis, 10 common peptides were identified among the proteins derived from tumor-bearing mice and those obtained from tumor-free mice (Table IV), while the remaining peptide fragments were unique in the tumor-bearing mice (Table V, n=22) and the tumor-free mice (n=25).

Among the peptides identified in tumor-free mice, twelve peptides were detected that partially or completely overlapped with thirteen peptides detected in mice with B16 melanoma (Table VI). A peptide fragment with an amino acid sequence of 104-121 and an m/z of 1,794.9294 was detected in tumor-free mice. This peptide completely overlapped with the peptide fragment with the amino acid sequence 111-119 and an m/z of 916.5098 found in mice with B16 melanoma. Furthermore, a peptide with the amino acid sequence of 140-158 and an m/z ratio of 2,415.1894 detected in tumor-free mice partially overlapped with a peptide with the amino acid sequence 140-159 and the m/z of 2,544.3711 found in mice with B16 melanoma. The peptide with the amino acid sequence of 171-195 and the m/z of 2,943.3268 detected in tumor-free mice completely overlapped with the peptide with the amino acid sequence 171-181 found in mice with B16 melanoma (m/z, 1,405.7664) and in tumor-free mice (m/z 1,405.7547). The peptide with the amino acid sequence of 265-273 and the m/z of 1,016.4871 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 267-273 and the m/z of 790.429 found in mice with B16 melanoma. The peptide with the amino acid sequence of 280-294 and the m/z of 1,666.8365 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 279-294 and the m/z of 1,780.0200 and completely overlapped with the peptide with the amino acid sequence of 280-289 and the m/z ratio of 1,040.5248 found in mice with B16 melanoma. The peptide with the amino acid sequence of 404-431 and the m/z of 3,202.485 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 404-424 and the m/z of 2,354.1529 from mice with B16 melanoma. The peptide with the amino acid sequence of 544-560 which was detected in tumor-free mice (m/z, 2,156.1239) and in mice with B16 melanoma (m/z, 2,156.0816) partially overlapped with the peptide with the amino acids sequence of 547-566 and the m/z of 2,226.2278 found in mice with B16 melanoma. The peptide with the amino acid sequence of 612-635 and the m/z of 2,586.3754 detected in tumor-free

Table VI. Overlapping peptides of the inter-alpha-trypsin inhibitor heavy chain 4 in tumor-bearing and in tumor-free mice.

Animals	Peptide ion (m/z)	Start-end	Amino acid sequence
Tumor-free mice	1794.9294	104-121	K.QYSAAVGRGESAGIVKTT.G
B16 melanoma	916.5098	111-119	G.RGESAGIVK.T
Tumor-free mice	2415.1894	140-158	K.ITFELIYQELLQRRLGMYE.L
B16 melanoma	2544.3711	140-159	K.ITFELIYQELLQRRLGMYEL.L + oxidation (M)
Tumor-free mice	2943.3268	171-195	K.HLQMDIYIFEPQGISILETESTFMT.P
Tumor-free mice	1405.7547	171-181	K.HLQMDIYIFEP.Q
B16 melanoma	1405.7664		
Tumor-free mice	1016.4871	265-273	A.PENLPTMSK.N
B16 melanoma	790.429	267-273	E.NLPTMSK.N
Tumor-free mice	1666.8365	280-294	I.DKSGSMSGKKIQQTR.E + oxidation (M)
B16 melanoma	1780.0200	279-294	V.IDKSGSMSGKKIQQTR.E + oxidation (M)
B16 melanoma	1040.5248	280-289	I.DKSGSMSGKK.I + oxidation (M)
Tumor-free mice	3202.485	404-431	R.EAINGQYSLFCLGFGFDVNYPFLEKMAL. + oxidation (M)
B16 melanoma	2354.1529	404-424	R.EAINGQYSLFCLGFGFDVNYP.F
Tumor-free mice	2156.0816	544-560	F.MERLWALLTIQQQLEQR.I
B16 melanoma	2156.1239	544-560	F.MERLWALLTIQQQLEQR.I
B16 melanoma	2226.2278	547-566	R.LWALLTIQQQLEQRISASGA.E
Tumor-free mice	2586.3754	612-635	V.GDGMQRLPLAAQAHPFRPPVRGSK.L
B16 melanoma	3499.9311	610-641	V.EVGDGMQRLPLAAQAHPFRPPVRGSKLMTVLK.G
Tumor-free mice	1791.8798	656-673	R.ASRQYIPPGFPGPPGPPG.F
B16 melanoma	1987.0943	659-678	R.QYIPPGFPGPPGPPGFPAPP.G
Tumor-free mice	881.4796	822-827	T.EYKWKK.T
B16 melanoma	1330.7948	826-837	W.KKTLFSVLPGLK.M
Tumor-free mice	1311.651	895-905	V.WEPPVEPDNTK.R
B16 melanoma	1609.8461	896-909	W.EPPVEPDNTKRTVK.V
Tumor-free mice	793.4153	910-916	K.VQGVDYL.A
B16 melanoma	1277.697	906-916	K.RTVKVQGVDYL.A

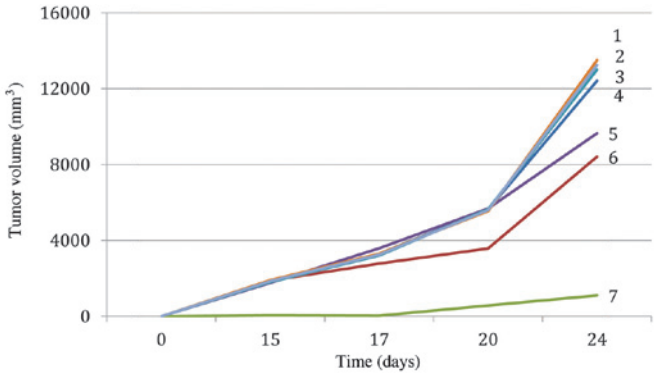
M, methionine.

Table VII. Interaction between albumin and the fatty acid spin probe 16-doxyl stearate acid determined by electron spin resonance analysis.

Parameter	Tumor-free mice	Tumor-bearing mice	P-value
Discrimination parameter	-3.08±0.06	-2.2±0.09	<0.05
Binding efficiency (%)	28.4±5.1	22.0±5.0	<0.05
Real transport quality (%)	39.9±5.4	34.5±6.3	>0.05
Detoxification efficiency (%)	14.6±4.5	9.7±3.8	>0.05

Data presented as the mean ± standard deviation, n>3. Statistical significance calculated using Student's t-test.

mice partially overlapped with the peptide with the amino acid sequence of 610-641 and the m/z of 3,499.9311 found in mice with B16 melanoma. The peptide with the amino acid sequence 656-673 and the m/z of 1,791.8798 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence 659-678 and the m/z 1,987.0943 found in mice with B16 melanoma. The peptide with the amino acid sequence of 822-827 and the m/z of 881.4796 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 826-837 and the m/z of 1,330.7948 found in mice with B16 melanoma. The peptide with the amino acid sequence of 895-905 and the m/z of 1,311.651 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 896-909



Tumor volume (mm^3) in groups

Day	1	2	3	4	5	6	7
0	0	0	0	0	0	0	0
15	1900	1850	1800	1790	1765,2	1875,4	50
17	3300	3250	3200	3300,8	3582,8	2782,4	29
20	5550	5600	5650	5619,2	5679,6	3579,6	569
24	13500	13250	13000	12413,25	9650,4	8413	1096

Figure 3. Influence of the inter-alpha-trypsin inhibitor heavy chain 4 and albumin on the growth of B16 melanoma in mice. The abscissa displays the days after transplantation of tumor cells and the ordinate displays the tumor volume (mm^3). Curves: (1) B16 melanoma growth in the control group; (2) B16 melanoma growth after injection of inter-alpha-trypsin inhibitor heavy chain 4, which was obtained from fresh blood serum of tumor-free C57Bl/6 mice; (3) B16 melanoma growth in mice after injection of albumin, obtained from fresh blood serum of C57Bl/6 mice with B16 melanoma; (4) B16 melanoma growth after injection of the animals with albumin obtained from fresh blood serum of tumor-free C57Bl/6 mice. (5) B16 melanoma growth after injection with inter-alpha-trypsin inhibitor heavy chain 4 obtained from frozen blood serum of C57Bl/6 mice with B16 melanoma. (6) B16 melanoma growth in mice after injection of inter-alpha-trypsin inhibitor heavy chain 4, which was obtained from the fresh blood serum of F1 hybrid mice (CBAxC57Bl/6) with B16 melanoma. (7) B16 melanoma growth in mice after injection of inter-alpha-trypsin inhibitor heavy chain 4, obtained from the fresh blood serum of C57Bl/6 mice with B16 melanoma. Injections of proteins were performed two weeks prior to tumor cell inoculation. Student's t-test used for analysis and n=10.

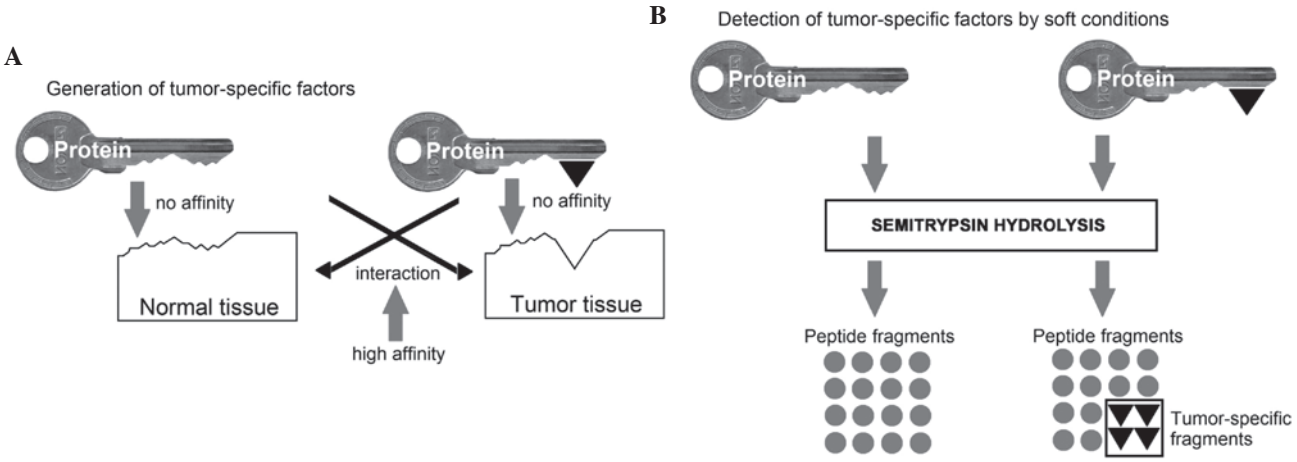


Figure 4. (A) Generation and (B) detection of tumor-specific factors.

and the m/z of 1,609.8461 found in mice with B16 melanoma. The peptide with the amino acid sequence of 910-916 and the m/z of 793.4153 detected in tumor-free mice partially overlapped with the peptide with the amino acid sequence of 906-916 and the m/z ratio of 1,277.697 found in mice with B16 melanoma. Thus, the same pattern to that observed for albumin was confirmed for ITIH4, i.e. ITIH4 obtained from mice with B16 melanoma provided semi-tryptic peptide fragments that differed from the semi-tryptic peptide fragments of ITIH4 from the serum of tumor-free mice. This result indicated a difference in the availability of proteolysis sites and therefore, a different molecular conformation of albumin and ITIH4 in the serum of tumor-bearing and tumor-free mice.

Serum albumin has differential conformation-depending binding activity between tumor-bearing and tumor-free mice. In the next stage of the present study, it was required to test whether the biological activity of the assessed proteins was altered upon their conformational changes. For the assessment of the functional activity of albumin, ESR analysis was used, allowing for the estimation of the conformational status and transport parameters of albumin. For this purpose, the interaction of albumin with the spin probe 16-doxyl stearic acid was examined. As the results in Table VII demonstrate, the spin-labeled acid interacted differently with albumin depending on whether it was obtained from tumor-free or tumor-bearing mice. The largest difference (almost 1.4 times) among all values determined was observed in the discrimination parameter.

According to the developers of the ESR device, this the discrimination parameter characterizes the conformation of the molecule. Therefore, the results obtained supported the hypothesis that albumin in tumor-bearing mice and tumor-free mice have differential conformations, which may be involved in the regulation of the tumor-associated activity of proteins.

Prior injection of ITIH4 from mice with B16 melanoma reduces melanoma growth in mice. Subsequently, a series of *in vivo* experiments were performed to study the influence of inter ITIH4 (serpin) and albumin on the growth of B16 melanoma (Fig. 3). For this purpose, mice were injected with albumin or ITIH4 from plasma from mice with B16 melanoma or tumor-free mice two weeks prior to tumor cell inoculation, and the effect on tumor growth was observed (4). It was discovered that albumin, regardless of cancer status of the animal from which it was obtained, had no effect on the growth of B16 melanoma in mice. Serpin obtained from fresh blood serum of tumor-free animals or from frozen blood serum of mice with B16 melanoma did not affect the growth of B16 melanoma. As shown in Fig. 3, a significant inhibition of the tumor growth was only achieved in group 7, in which animals had been injected with ITIH4, which was obtained from fresh serum of C57Bl/6 mice with B16 melanoma, prior to transplantation of the tumor. Prior freezing of this serum or the use of allogeneic protein led to the loss of the tumor-specific activity. Thus, only the protein extracted from fresh blood serum of C57Bl/6 mice significantly inhibited the growth of melanoma in C57Bl/6 mice.

The inhibition of the tumor growth by ITIH4 protein was reflected by the lengthening of the period until the visual appearance of the tumor: In the mice of the control group, the appearance of the tumor was noted 10 days after tumor transplantation, whereas in the experimental group, the tumors were registered only at day 20 after tumor cell transplantation. This may be interpreted as 100% tumor growth inhibition by ITIH4 over 10 days. The lifespan of animals with B16 melanoma in the control group averaged 40.8±2.1 days, whereas in group 7, in which ITIH4 was injected, it was 65.3±3.4 days ($P<0.05$).

Discussion

To date, the biological effects of serum proteins and their regulation have not been fully elucidated. In the present study, the tumor-specific activity of serum proteins was assessed, as well as their regulation by conformational changes. However, the underlying mechanisms of this effect have yet to be elucidated. In addition, it was not possible to reproduce the *in vivo* results in an *in vitro* system; this may be due to the metabolism and homeostasis in a living organism, which cannot be reproduced by an *in vitro* cell model.

A previous study by our group demonstrated the tumor-specific activity of the blood serum fractions of mice with Ehrlich carcinoma, which contained proteins with a molecular weight of 50-100 kDa (4). This fraction contained 40 proteins and differences were most obvious by electrophoretic mobility assay in the major band with a molecular weight of ~65 kDa. The major changes in this band coincided with the disappearance of alpha-1-anti-trypsin and the appearance in the same sample of cathepsin L1. Inter-α-trypsin inhibitor was additionally identificated in this fraction. This led to the hypothesis that the tumor-specific activity of serpin is exerted by α-1-anti-trypsin, even though the molecular weight of this protein is significantly lower and was 45 kDa. In the present study, a protein with a similar molecular weight of 65-70 kDa from the serum of mice with B16 melanoma, identified as serpin ITIH4, also showed a specific activity. The results of the previous studies by our group as well as those obtained in the present study suggested that in the two cases - Ehrlich carcinoma and B16 melanoma - the protein with a tumor-specific activity was identical and that its conformation determined its biological specificity.

In previous studies, our group as well as other researchers, did not identify any significant differences in the spectrum of proteins isolated from the serum of tumor-bearing and tumor-free mice (3,4). In spite of this, these blood serum proteins were observed to have a tumor-specific activity. This fact can be explained by differences in sample preparation of these proteins in the previous and present studies. In particular, in prior studies, at least two methods were used which cause the denaturation of proteins (prior freezing of samples or boiling in a buffer for electrophoresis). The denaturation facilitates proteolysis and the identification of proteins, but prevents the evaluation of features of their conformation.

Therefore, in the present study, the protein conformation was not investigated using these standard methods. Only fresh serum samples were used, the separation of proteins was performed by column chromatography and the changes in conformation of the proteins were estimated via the soft proteolysis (with 10% acetonitrile) product. It was discovered that identical sites of the same protein yielded differential peptide fragment spectra between tumor-free mice and those with melanoma. This result demonstrated the difference in the availability of these sites for the enzymes, and hence, the differential conformation of the proteins between the two experimental groups. It is important to note that the conformational changes of the proteins studied were associated with changes in their biological activity. Thus, the present study demonstrated for the first time, to the best of our knowledge, that the tumor-specific activity of ITIH4 was based on its differential conformation between tumor-free mice and those with melanoma. This result confirmed the hypothesis that the conformation of serpin determines its tumor-specific effect.

The results obtained can be figuratively explained as illustrated in the scheme in Fig. 4A: Serum protein can be regarded as a key, which has a stable part and a specific component. The stable part, represented in the present study by common peptides, is present in all organisms of the given species. In the present study, the specific component is constituted by unique peptides in a particular individual and is defined by its state, in particular, by the presence of a tumor. The main condition for the functioning of this protein (key) is the lack of high affinity to the tissues of the body and other serum proteins. Tumor growth leads to an increased concentration of these groups of antigenic determinants, which are located on the surface of the tumor cells. In response to this increased concentration of groups of antigenic determinants in cells, the availability of antigenic determinants, which are capable of interacting with them,

are expected to decrease in the serum. This may be achieved in two ways: The emergence of acute phase proteins among the blood serum proteins (5), and conformational changes in serum proteins, as shown in the present study. In order to study this adaptation to tumor growth, the present study performed a proteomics study, which required proteases to cleave the native protein. Conventional methods of proteomic analyses of proteins include the preliminary freezing of the samples and boiling for electrophoresis. However, in the present study, this denaturation resulted in the loss of the tumor-specific activity of the samples. Therefore, the conditions of sample preparation were modified by excluding any experimental conditions leading to protein denaturation (Fig. 4B).

In conclusion, the results of the present study confirmed the hypothesis that the conformation of serum proteins is associated with their biological activity and with tumor growth in the body. Injection of ITIH4 from the serum of mice with melanoma was demonstrated to inhibit tumor growth in a mouse model of melanoma under the condition that its conformation was preserved.

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