Structural Changes in Albumin Are a Possible Mechanism for Fluctuation of Cefazorin and Ibuprofen Plasma Protein Binding in Rats with Carcinogen-induced Osteosarcoma

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Abstract. *Background/Aim: It is known that the protein-unbound fraction (fp) of warfarin fluctuates in the plasma of cancer patients and the fluctuation of fp is correlated with albumin concentration. However, this mechanism remains unclear. The present study was performed with the objective of elucidating variations in the protein-binding rate of specific drugs in the presence of cancer, as well as the mechanisms involved. Materials and Methods: Experiments were performed using Fisher 344 model rats, that we have used in previous studies. A single i.v. injection of cefazolin (CEZ) 20 mg/kg or ibuprofen (IB) 10 mg/kg was administered to both tumor-bearing and control groups. We compared relevant pharmacokinetics. Purified albumin was checked for purity with SDS-PAGE and was used in experiments on protein binding of CEZ and IB. Results: The fp of CEZ in plasma from the tumor-bearing group increased approximately 2.9-fold and the fp of IB also increased about 2.7-fold. For that reason, we purified albumin from plasma and examined its spectroscopic signature. We showed that conformational changes (i.e., decreases in the hydrophobic region, increases in endogenous tryptophan fluorescence intensity and decrease in α-helical content) had occurred in albumin in the tumor-bearing group. Conclusion: In the present study, we confirmed that, in a state of cancer morbidity, fp was elevated for site I high-affinity CEZ and site II high-affinity IB. Moreover, our findings indicated that, as a cause of those elevations, a decrease in the albumin concentration and conformational changes due to an oxidation are involved.*

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The potency of pharmacological effects *in vivo* depends greatly on the amount of free-form of a drug that migrates to the target tissue(s). Recently, the importance of evaluating protein-unbound drug forms has been noted, even on the basis of pharmacokinetic-pharmacodynamic (PK-PD) theory. One of the main regulatory factors is albumin. Albumin is a protein present in large amounts in plasma and constitutes about 60% of the total protein in plasma. Albumin is known to decrease in cases of renal disorder, hepatic disorder and inflammatory diseases, such as rheumatism and cancer (1).

Albumin binds mainly to acidic drugs. Its binding sites are I and II, which were discovered by Sudlow *et al.* (2). Albumin is a simple protein composed of 585 amino acids with a molecular weight of 65 kDa. Its basic structure is three-dimensional and is made up of three highly homologous domains. In aqueous solution, the three domains take on a folded structure, with each of the domains in close proximity. It is estimated that the entire molecule takes on a

Table I. *Estimated pharmacokinetic parameters after cefazolin (CEZ) 20 mg/kg or ibuprofen (IB) 10 mg/kg i.v. bolus injection for control and tumor-bearing rats.*

Drug	Pharmacokinetic parameters	Control rats	Tumor-bearing rats
CEZ	CLtot (ml/min/kg)	2.93±0.67	5.13±1.20*
	Vss (ml/kg)	214±26	314±32*
IB	CLtot (ml/min/kg)	8.05±0.01	6.25±1.24
	Vss (ml/kg)	539±66	1015±290*

Each value is the mean±SD (n=3-5). *Significant difference between control and tumor -bearing rats at $p<0.05$.

Table II. *Laboratory data of the indicated parameters in plasma for control and tumor-bearing rats.*

Parameters	Control rats	Tumor-bearing rats
Total protein (g/dl)	5.28±0.12	5.33±0.22
Albumin (g/dl)	3.89 ±0.10	3.46±0.48*
Albumin/globulin	2.83±0.27	1.92±0.37*
Bilirubin (mg/dl)	0.65±0.06	0.69±0.06
Free fatty acids (μEQ/l)	621±53	551±218
Glucose (mg/dl)	240±31	213±22
Creatinine (mg/dl)	0.230±0.047	0.297 ±0.097
Uric acid (mg/dl)	2.29±1.37	1.95±0.85

Each value is the mean±SD (n>6). *Significant difference between control and tumor-bearing rats at $p<0.05$.

heart-shaped form. Each domain also has two sub-domains and has a single tryptophan residue at position 214. This tryptophan is located in the central area of subdomain IIA. Binding site I is located in the hydrophobic pocket of subdomain IIA. Typical ligands are bulky heterocyclic anions, such as cefazolin (CEZ) and warfarin. A compound with an electric charge is in the central portion of these molecules (3). Site II is located in domain IIIA and the typical ligands for site II are ibuprofen (IB) and diazepam. The extended structure of these aromatic carboxylic acids has a charge at the end of the molecule. It is known that the protein-unbound fraction (fp) of warfarin fluctuates in the plasma of cancer patients and this fluctuation is correlated with albumin concentration (4). However, the precise mechanism remains unclear. Liliemark *et al*. showed significantly higher *in vivo* protein binding rates for etoposide in children, compared to adult cancer patients. The correlation coefficient of the free concentration of etoposide with albumin concentration was reduced to 0.26. It was thought that there must be factors, other than albumin and bilirubin, involved (5).

In addition, Shimada *et al*. investigated the pharmacokinetics of vancomycin in rats with carcinogen-induced osteosarcoma and reported significantly enhanced systemic and renal clearance of the drug in the tumor-bearing group. They examined plasma protein binding rate as a causative factor and found that the protein-binding rate of vancomycin was 30%. The fp of vancomycin showed no variation (6). *In vivo*, the roles of albumin include regulation of colloid osmotic pressure, anti-oxidant action, binding and transport of endogenous (fatty acids, bilirubin and uremic substances) and exogenous (drugs) compounds (7).

The present study was performed with the objective of elucidating variations in the protein-binding rate of specific drugs in the presence of cancer, as well as the mechanisms involved. Experiments were performed using the rat Fisher 344 model, that we have used in previous studies (6).

Table III. *Advanced Oxidation Protein Products (AOPP) level of plasma and purified albumin from control and tumor -bearing rat's plasma.*

Relative antioxidant activity		Control rats	Tumor-bearing rats
AOPP	Rat naive	1.51±6.1	32.5±6.8*
(mM/mg protein)	Rat purified	2.08±1.93	14.0±2.5*
Carbonyl	Rat purified	1.28±0.23	2.99±0.27*
(nmol/mg protein)	HSA naive	2.30±0.37	-
	HSA+CT 10 mM	21.73±1.56†	-

CT: Chloramine T; Each data is mean±SD (n=3). *Significant difference between control and tumor -bearing rats at $p<0.05$. †Significantly difference between human serum albumin (HSA)-naive and HSA-added CT (10 mM) at $p<0.05$.

Materials and Methods

Cefazolin sodium hydrate was purchased from Astellas Pharma Inc. (Tokyo, Japan). IB, dihydrorhodamine 123 and albumin from human serum, essential fatty acid-free were purchased from Sigma Chemical Co. (Tokyo, Japan). Chloramine T was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All reagents were of analytical grade.

Preparation of the tumor model. All animal experiments were carried out in accordance with the Declaration of Helsinki and with the Kanazawa University Guide for the Care and Use of Laboratory Animals. We adopted rat animals, using chemical carcinogen-induced osteosarcoma, selected lung metastatic lesions (C-SLM), transplanted into thigh muscles of 7-week-old, male Fisher 344 rats. When tumor size reached approximately 1,000 mm^3 11-12 weeks old), those rats were applied to the following experiment. Control animals were handled in the same manner, except that no tumor cells were implanted (6).

Pharmacokinetics. A single *i.v.* injection of CEZ, 20 mg/kg, or IB, 10 mg/kg, was administered to both tumor-bearing and control groups. Following injection, blood was collected in aliquots of about 0.45 ml from the left carotid artery at designated times. Assay methods were essentially the same as those reported by Deguchi *et al*. (8) for CEZ and by Satterwhite *et al*. (9) for IB. The plasma

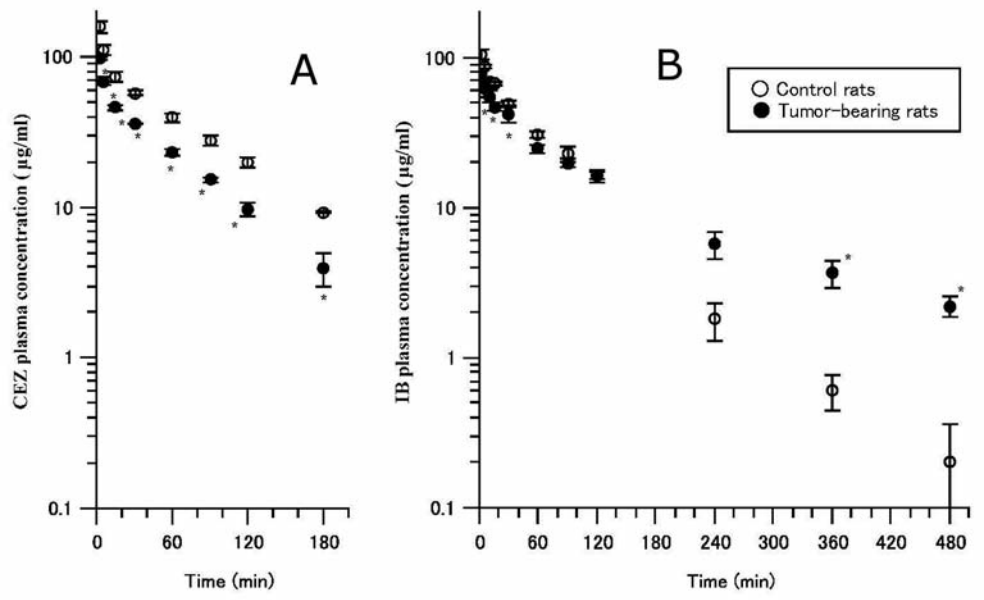

Figure 1. *Plasma concentration time courses of CEZ 20 mg/kg (A) or IB 10mg/kg (B) i.v. bolus injection for control (open) or tumor-bearing rats (closed symbol). *Each point with vertical bar represent the mean±SEM (n=3-5). *Significantly different from the control at p<0.05.*

concentration data after *i.v.* administration of CEZ or IB were analyzed by a non-compartment method (10).

Protein binding. For protein-binding experiments, CEZ or IB solution was added to 700 μl of either untreated or active carbon-treated plasma. After ultrafiltration (Centrefree; Millipore Corporation, Bedford, MA, USA,) the filtrates were assayed by HPLC.

Plasma endogenous substances affecting plasma protein binding. Tumor-bearing and control groups were compared for the following: total protein concentration, albumin concentration, albumin/globulin ratio, bilirubin level, free fatty acid concentration, glucose level, creatinine level and uric acid level. Reference is made to Shimada *et al*. for assay methods for plasma endogenous substances. Total protein and albumin were assayed by BCG (Bromcresol Green). Total bilirubin was measured by using vanadic acid. Creatinine and free fatty acid were measured by enzyme assay. Glucose was assayed by hexokinase UV method. Uric acid was assayed by uricase POD method.

Purification of albumin from tumor-bearing group plasma. Albumin was purified on a Hitrap Blue HP column (GE Healthcare Japan, Tokyo, Japan) while monitoring the absorption at 280 nm. Purified albumin was checked for purity with SDS-PAGE and was used in experiments on protein binding of CEZ and IB.

Albumin spectrophotometric analysis. Measurement of the antioxidant capacity of albumin and the hydrophobicity evaluation of albumin using (4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid, Dipotassium Salt) (bis-ANS) was recorded on a Jasco FP7700 apparatus (Jasco, Tokyo, Japan) according to the methods of Mera *et al*. (11). The fluorescence spectrum excitation wavelength was set at 394 nm and the fluorescence of bis-ANS was measured at 400~600 nm. The rate of oxidation of dihydrorhodamine 123 to rhodamine 123 was evaluated as the antioxidant capacity of albumin by monitoring the fluorescence spectrum at 525 nm for 3 min. The fluorescence spectrum was measured by excitation at 280 or 295 nm for purified albumin. The circular dichroism (CD) measurements were performed using a J-820 spectropolarimeter (Jasco, Tokyo, Japan) according to Mera *et al*. (10).

Assay of oxidative stress markers. The antioxidant capacity of albumin was measured in accordance with the method of Revine *et al*. by measuring the carbonyl content, an oxidative stress marker (12). A second oxidative stress marker, advanced oxidant protein products (AOPP), was assayed in accordance with the method of Witkko-Sarsat *et al*. (13). Albumin was treated with 10 mM chloramine T to yield oxidized albumin and its spectroscopic characteristics and drug-binding capacity were examined.

Statistical analysis. The student's *t*-test was used to compare the unpaired mean values of two sets of data. The number of determinations is noted in each Table and Figure. A p-value <0.05 indicated a statistically significant difference between data sets.

Results

Comparison of pharmacokinetics. Figure 1A shows the plasma concentration time-course data following bolus *i.v.* injection of CEZ, 20 mg/kg. The concentration in plasma decreased with time in both animal groups. The concentration in the tumor-bearing group was 1.8-times lower than in the control group. Figure 1B shows the plasma concentration time-course data following bolus *i.v.* injection of IB, 10 mg/kg. The concentration in plasma was significantly lower in the tumor-bearing group than in the control group at 2, 5, 15 and 30 min after injection, but was significantly higher at 360 and 480 min.

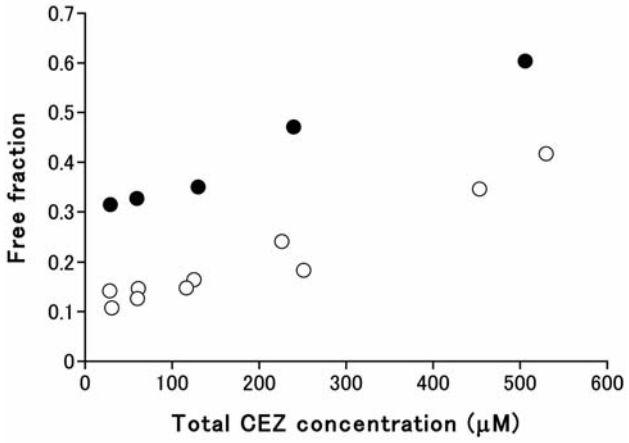

Figure 2. *Relationship between free fraction of CEZ and total CEZ concentration with control (open) or tumor-bearing (closed symbol) rat's plasma. *Significant difference between control and tumor-bearing rats at p<0.01.*

Protein binding. Table I shows the pharmacokinetic parameters obtained for CEZ. The mean systemic clearance (CL_{tot}) (ml/min/kg) was 2.93±0.67 in the control group and 5.13±1.20 in the tumor-bearing group. The tumor-bearing group value was elevated 1.8-fold over that in the control group. Distribution volume (Vss) (ml/kg) showed mean values of 214±26 in the control group and 314±32 in the tumor-bearing group. The value in the tumor-bearing group was 1.5-fold higher than that in the control group. Figure 2 shows the fp data; mean values were 0.247±0.077 in the control group and 0.722±0.111 in the tumor-bearing group. The protein-unbound fraction changed 2.9-fold. Mean Vss was 539±66 in the control group and 1015±290 in the tumor-bearing group.

Vss was significantly elevated by approximately 1.9-fold. Table I also compiles the pharmacokinetic parameters that were obtained for IB. No significant changes were seen in CL_{tot}, but mean Vss was 539±66 in the control group and was significantly elevated by approximately 1.9-fold to 1015±290 in the tumor-bearing group. For IB, fp was 0.026 in the control group and 0.069 in the tumor-bearing group. This was significantly higher by approximately 2.7-fold in the tumor-bearing group.

Plasma endogenous substances affecting plasma protein binding. Table II shows the results for other plasma endogenous substances that affect albumin and plasma protein binding. Albumin was significantly decreased by approximately 10% in the tumor-bearing group and free fatty acids were also decreased by about 10%. No difference was observed in total protein concentrations between the control and tumor-bearing group but the albumin/globulin ratio was significantly decreased in the tumor-bearing group. No significant changes were seen in bilirubin, glucose, creatinine or uric acid levels.

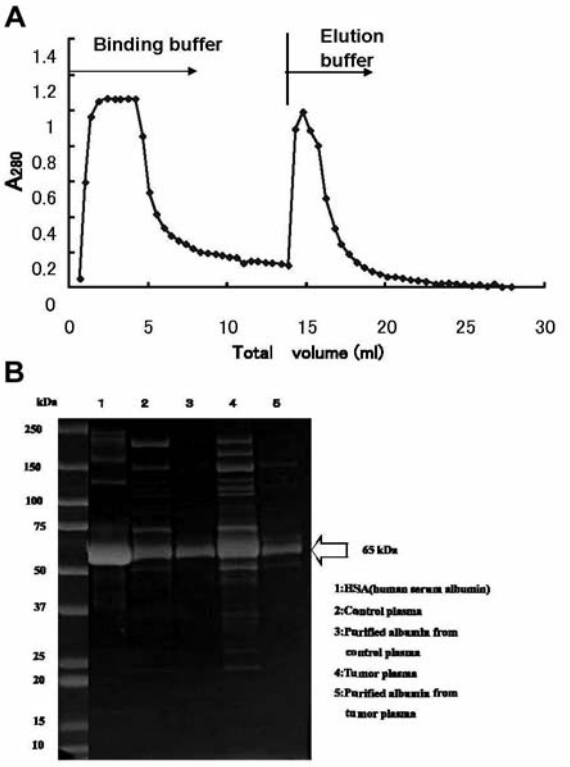

Figure 3. *Characteristics of purified tumor-bearing rat's plasma; Panel A: Separation of rat serum albumin from rat plasma; Panel B: SDS-PAGE of human serum albumin (HSA), purified albumin and plasma.*

Albumin purification from tumor-bearing rats' plasma. We decided to examine the albumin structural change of tumor-bearing rats. For this reason, albumin was purified from the tumor-bearing group plasma. Figure 3 shows the results of purity analysis of purified albumin by SDS-PAGE. The purity of the band indicated by the arrow was approximately 75% human serum albumin (HSA). The control plasma was about 56%. After purification of control plasma, purity was about 88%. Purity was about 42% for the tumor-bearing group plasma, while after purification of the tumor-bearing group plasma, purity was about 75%. Albumin purity in the purified group was increased by at least 30% when compared to plasma albumin. It was also confirmed that the purified albumin was higher in purity than HSA. In the control group, the purity of the band in the vicinity of 65 kDa was high in both plasma and purified albumin. It was confirmed that the tumor-bearing group contained more bands than the control group. This trend was pronounced for polymers. Protein binding of CEZ and IB was performed using purified albumin. The fp of CEZ was 0.63±0.05 in the control group and 0.73±0.04 in the tumor-bearing group. At low concentrations, the fp of CEZ tended to increase, whereas at high concentrations it was about the same in the

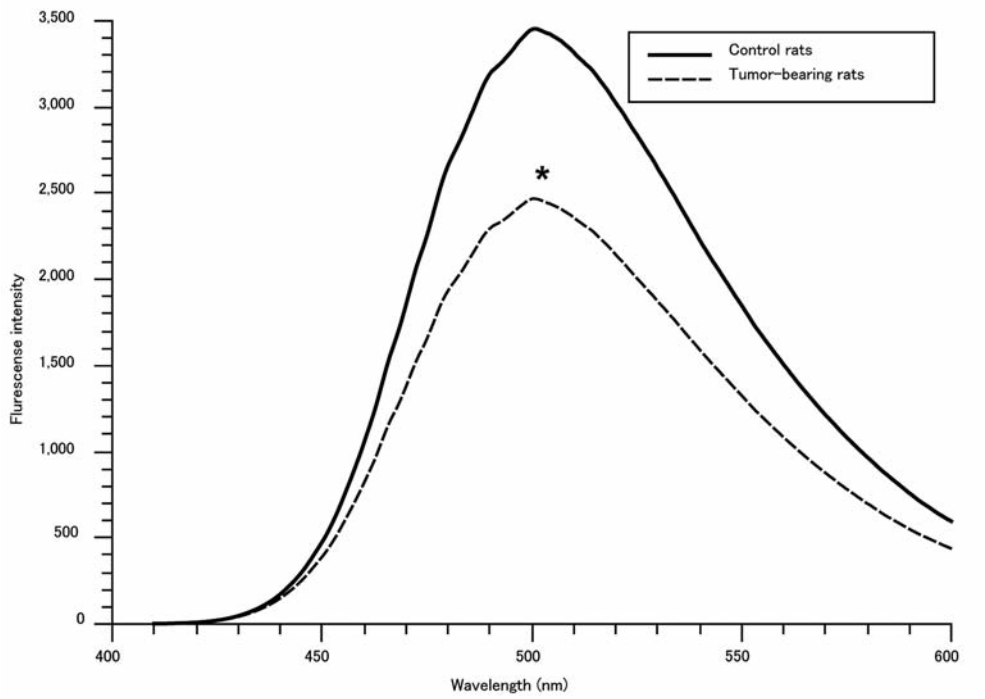

Figure 4. *Fluorescence of albumin-bound bis-ANS in control and tumor-bearing rat's plasma. Each data is mean±S.D. (n=6). *Significant difference between control and tumor -bearing rats at p<0.05.*

control and tumor-bearing groups. The fp of IB was 0.07±0.03 in the control group and 0.19±0.02 in the tumor-bearing group. With IB, fp was significantly elevated, approximately 2.7-fold, in the tumor-bearing group, while the results were similar with plasma.

Spectrophotometric analysis of albumin. Figure 4 shows the results for variations in fluorescence intensity of bis-ANS using purified plasma. Bis-ANS is a commonly used marker for the hydrophobicity of albumin. Binding of bis-ANS was significantly reduced in plasma from the tumor-bearing group, indicating that the hydrophobic region was decreased. Radiation at 280 nm excites albumin purified from control or tumor-bearing group plasma. Figure 5A shows that the intensity of fluorescence was significantly increased in the tumor-bearing group. This means that both tyrosyl and tryptophyl residue fluorescence was different between control and tumor-bearing group (14). In addition, Radiation at 295 nm excites albumin purified from control or tumor-bearing group plasma. Figure 5B shows that the intensity of fluorescence was significantly increased in the tumor-bearing group. It means that tryptophyl residue fluorescence was different in control and tumor-bearing groups. The estimated α-helix contents from CD spectra of purified albumin obtained from control or tumor-bearing rat plasma were 0.59±0.008 and 0.44±0.01, respectively.

Assay of oxidative stress markers. One of the physiological roles of albumin is its anti-oxidative function in relation to active oxygen in plasma. We investigated these anti-oxidative effects using plasma, with the degree of oxidation of albumin itself used as an index. We added hydrogen peroxide, which is a form of active oxygen, to diluted serum. Figure 6 shows the results of measurement of the rate of oxidation of dihydrorhodamine 123 (DRD) to rhodamine 123 (RD) performed by monitoring the fluorescence intensity spectrum. The results showed that the oxidation rate was significantly accelerated when plasma from the tumor-bearing group was added. These results indicate that the anti-oxidative effects were decreased. Table III shows the results of measurement of Advanced Oxidation Protein Products (AOPP), which is a marker of protein oxidative stress. The concentration of AOPP activity was converted to the corresponding amount of chloramine T, which is a representative oxidized substance, and corrected for the protein concentration. The results show that the activity was 15.1 mM/mg protein in the control plasma, compared with 32.5 mM/mg protein in the tumor-bearing group plasma. The activity in the tumor-bearing group plasma was significantly elevated approximately 2-fold over control group activity. AOPP was also assayed for albumin that had been purified from plasma. Activity was 2.1 mM/mg protein for the control group, as compared to 14.0 mM/mg protein for the tumor-bearing group. The activity for

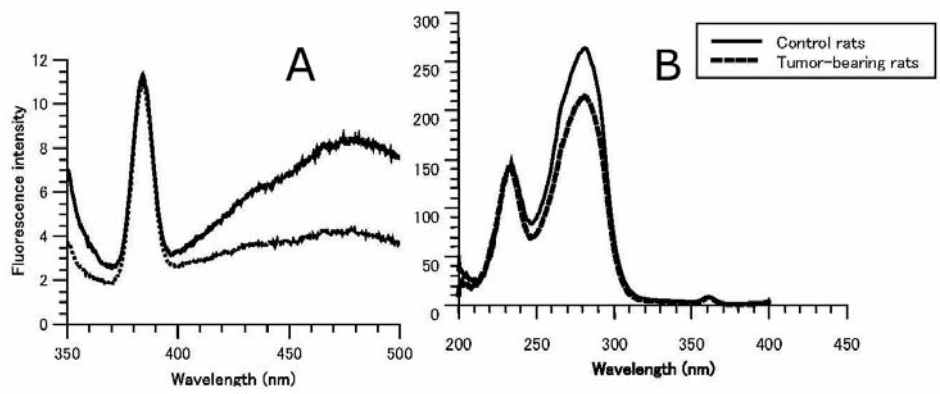

Figure 5. *Spectroscopic analysis of purified albumin from control and tumor bearing rat's plasma. Fluorescence intensity of aromatic series by excitation at 280 nm (A) and tryptophan by excitation at 295 nm (B). Data are mean±S.D. (n=3). *Significant difference between control and tumor-bearing rats at p<0.05.*

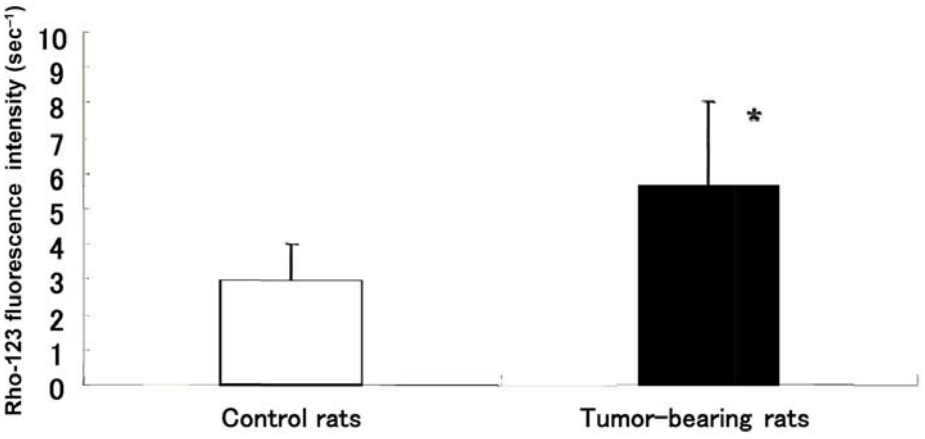

Figure 6. *Effect of relative antioxidant activity between control and tumor bearing rat serum in oxidative rate from dihydroRho123 to Rho123.-Each data is mean±S.D. (n>9) *Significant difference between control and tumor -bearing rats at p<0.05.*

the tumor-bearing group was significantly elevated approximately 7-fold over the control group.

Another protein oxidative stress marker is the carbonyl content, that we assayed using purified albumin. These results are also presented in Table III. The control group showed a value of 1.3 nmol/mg protein, while the value for the tumor-bearing group was 3.0 nmol/mg protein, which was significantly elevated approximately by 2.3-fold to the control group. The carbonyl content in oxidized albumin that had been treated with chloramine T, 10 mM, was elevated almost 10-fold to the control values.

Discussion

The fp of CEZ in plasma from the tumor-bearing group increased approximately by 2.9-fold and the fp of IB also increased by about 2.7-fold. As causative factors, we compared the concentrations of various endogenous substances. Albumin decreased by about 10% (3.9 g/dl *vs.* 3.5 g/dl) in the tumor-bearing group. However, no significant changes were seen for bilirubin, fatty acids or creatinine. If fp was constant less than 100 μM CEZ total concentration, total albumin concentration of tumor-bearing rats ($P_t$tumor) were estimated by following equation.

$$P_t^{tumor} = P_t^{control} \frac{1/fp^{tumor} - 1}{1/fp^{control} - 1}$$

where fp^{tumor} and $fp^{control}$ denote unbound fraction of tumor or control rat, respectively, and $Pt^{control}$ represent total albumin concentration of control rat. Therefore, using fp^{tumor}, $fp^{control}$, $Pt^{control}$ as 0.366, 0.158, 3.9 g/dl, Pt^{tumor} was estimated about 1.3 g/dl in the tumor-bearing group to explain the increase in the fp of CEZ only due to the decrease in albumin concentration, indicating the possibility

that conformational changes in albumin contribute to the increased fp. For that reason, we purified albumin from plasma and examined its spectroscopic signature. We showed that conformational changes (*i.e.*, decreases in the hydrophobic region, increases in endogenous tryptophan fluorescence intensity and decrease in α-helical content) had occurred in albumin in the tumor-bearing group. However, the causes of these conformational changes remain unclear.

In general, active oxygen species increase in the presence of cancer. This creates a state in which the balance of defense mechanisms against oxidative stress is perturbed and albumin becomes the main target in the plasma. When we investigated oxidative stress in the plasma, the tumor-bearing group showed a decrease in antioxidant capacity and elevated AOPP levels. Moreover, even purified albumin showed elevated AOPP levels in the tumor-bearing group, while carbonyl content was also elevated. Therefore, albumin was in a more highly oxidized state in the tumor-bearing group than in the control group.

Next, we prepared an artificial model of oxidized albumin, investigated its spectroscopic characteristics and drug-binding capacity and compared the findings with albumin that had been purified from plasma from the tumor-bearing group. The results showed that the hydrophobic region and the α-helical content of the oxidized albumin tended to be decreased. These findings were similar to the tumor-bearing group. We also reproduced the finding that fp was elevated for both CEZ and IB.

In this study, we confirmed that, in a state of cancer morbidity, fp was elevated for site I high-affinity CEZ and site II high-affinity IB. Moreover, our results indicated the presented findings as a cause of those elevations.

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