Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 552-557

PURIFICATION OF A SERUM DNA BINDING PROTEIN(64DP) WITH A MOLECULAR WEIGHT OF 64,000 AND ITS DIAGNOSTIC SIGNIFICANCE IN MALIGNANT DISEASES

Tsunehiko Katsunuma, Michio Tsuda, Takashi Kusumi, Tomoichi Ohkubo, *Toshio Mitomi,*Hisao Nakasaki,*Tomoo Tajima,*Seihichi Yokoyama, **Hiroshi Kamiguchi,***Kazuo Kobayashi and****Hirotaka Shinoda

Department of Biochemistry,*Department of Surgery,**Biochemistry Division of Interdepartmental Laboratory,***Department of Obstetrics and Gynecology, School of Medicine, Tokai University, Bohseidai, Isehara, 259-11, Japan, and ****Research Institute, Kyorin Pharmaceutical Co., LTD., Tokyo, Japan.

Received February 5,1980

Summary: A DNA binding protein with a molecular weight of 64,000(64DP) has been purified to homogeneity from human serum, and its quantitative assay has been developed. The average level of serum 64DP in 30 normal controls was 41.4 μg/ml, whereas it was 175 μg/ml in 87 patients with untreated malignant disease. Furthermore it was found to be elevated in all tested patients, 8 cases, with carcinoma in early stages. Serum 64DP has been found to be different from C3DP, CEA[#] or α-FP[#], and it appears that this protein might prove to be a useful tumor marker in malignant diseases.

The presence of a serum DNA binding protein(C3DP) was previously described in association with human malignant disease(1-4). We have isolated another human serum DNA binding protein, characterized as a monomeric protein with a molecular weight of 64,000(64,000 DNA Binding Protein: 64DP), different from human complement C3 or C3DP. The present report describes the method of purification of 64DP and the promising diagnostic value of this previously unexplored serum protein in cancer patients.

MATERIALS AND METHODS

Assay for 64DP: We have developed a quantitative assay system of serum 64DP level. All solutions contained 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 mM PMSF[#]. Each serum sample(1 ml) was applied to a DEAE Sephadex A-50 column (0.9 x 3 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 6.5, containing 150 mM NaCl. The column was washed with 50 ml of the same buffer and eluted with 18 ml of 10 mM potassium phosphate buffer, pH 6.5, containing 500 mM NaCl. This fraction was dialyzed against 10 mM potassium

[#]Abbreviations: CEA, carcinoembryonic antigen; α-FP, α-fetoprotein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

0006-291X/80/060552-06$01.00/0

phosphate buffer, pH 6.8, and applied to a DNA cellulose column(0.9 x 1.5 cm) containing 0.33 g of DNA cellulose equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The column was washed with 20 ml of the same buffer and eluted with 8 ml of 10 mM potassium phosphate buffer, pH 6.8, containing 500 mM NaCl. To this fraction, ammonium sulfate was added to 70% saturation and the mixture was centrifuged at 10,000 xg for 20 min. The precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer. Twenty one μg of protein was modified with SDS[#] and applied to SDS polyacrylamide gel. SDS polyacrylamide gel electrophoresis was carried out using the system of Laemmli(5). After electrophoresis, the gel was stained using the procedure of Fairbanks et al(6) and the amount of 64DP was determined by scanning method at 645 nm by using Helena densitometer using bovine serum albumin as a standard.

Immunochemical studies---Difference from C3DP, CEA and α-FP: C3DP was purified by the method of Parsons and Hoch(2). Antibodies to C3DP and to 64DP were prepared by giving rabbits subcutaneous injections of C3DP or 64DP protein (each containing 3 mg protein) in complete Freund's adjuvant, boostimg them similarly 2 weeks later. One week after the second injection, 20-40ml of blood was collected and clotted. Antisera of C3DP and of 64DP were obtained from the clots following centrifugation. 64DP and C3DP with their respective antisera were tested in Ouchterlony double diffusion studies. Radioimmunoassay for CEA and α-FP were performed on the ammonium sulfate fractions obtained during 64DP assay of sera with known elavated CEA or α-FP.

Clinical application of 64DP:Serum 64DP levels were determined in 30 healthy controls, in 10 umbilical cord blood samples, in 27 patients with non-malignant diseases and in 87 patients with various malignant diseases.

RESULTS AND DISCUSSION

DNA binding proteins: The DNA binding proteins in human serum were separated by DEAE Sephadex chromatography, affinity chromatography on DNA cellulose and SDS polyacrylamide gel electrophoresis, as described in "Materials and Methods". As shown in Table I, examination of the DNA binding proteins in sera from normal individuals and patients with malignant diseases showed that the amounts of some DNA binding proteins were elevated in the patients with malignant diseases compared with normal individuals. We examined the protein with molecular weight of 64,000 in the expectation that it was a previously unexplored protein, different from C3DP.

Purification of 64DP: All solutions contained 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 mM PMSF, and all procedures were performed at 4°C. Human serum from normal individuals(200 ml) was dialyzed against 10 mM potassium phosphate buffer, pH 6.5, containing 150 mM NaCl and applied to a DEAE Sephadex A-50 (3.6 x 21 cm) equilibrated with the same buffer. The column was washed the same buffer and then eluted with 10 mM potassium phosphate buffer, pH 6.5,

Table I.

The DNA Binding Proteins in Human Serum

M.Wt	Normal 1. (μg/ml)	Normal 2. (μg/ml)	[1]Cancer 1. (μg/ml)	[2]Cancer 2. (μg/ml)
28,000	22	18	71	77
33,000	24	27	41	49
40,000	6	2	13	4
41,000	14	7	31	33
64,000	59	46	156	238
74,000	70	59	189	136
80,000	46	52	263	232
90,000	43	49	124	121
111,000	19	16	35	28
140,000	40	25	33	43
170,000	24	13	59	39

[1]Cancer 1.: Serum from the patient with untreated stomach cancer.
[2]Cancer 2.: Serum from the patient with recurred uterus cancer.

containing 225 mM NaCl. The eluate was dialyzed overnight against 10 mM potassium phosphate buffer, pH 6.8. The solution was applied to a DNA cellulose column(3.4 x 4 cm) equilibrated with the same buffer. The column was washed with the same buffer and with 10 mM potassium phosphate buffer, pH 6.8, containing 100 mM NaCl. The column was eluted with 10 mM potassium phosphate buffer, pH 6.8, containing 300 mM NaCl. The eluate was subjected to ammonium sulfate fractionation(50-70% saturation). The precipitate was dissolved in the minimum volume of 10 mM Tris-HCl buffer, pH 7.8, containing 100 mM KCl and applied to a Sephadex G-150 column(1 x 95 cm) equilibrated with the same buffer. 64DP protein was eluted, appearing as a large single peak behind the leading peak of protein. Seven mg of purified 64DP was thus obtained from 200 ml of human serum from normal individuals.

Homogeneity: The final purified preparation showed a single band on disc electrophoresis in 10% polyacrylamide gel(pH 8.6). Homogeneity of the protein was also examined in SDS polyacrylamide gel electrophoresis with 0.6 M 2-mercaptoethanol and the molecular weight was estimated as to be 64,000, using rabbit muscle phosphorylase, bovine serum albumin, beef liver glutamate

dehydrogenase, ovalbumin, hog muscle lactate dehydrogenase and bovine erythrocyte carbonic anhydrase as markers. Sedimentation equilibrium studies(HITACHI 282 ultracentrifuge) were performed at three different protein concentrations (0.31, 0.39 and 0.42 mg/ml in 10 mM Tris-HCl buffer, pH 7.8, containing 100 mM KCl), at 10,000 rev./min, and the molecular weight was estimated as to be 64,000(assumed $\bar{v}$=0.75 g/liter). This value is in good agreement with the data obtained by SDS polyacrylamide gel electrophoresis, indicating the absence of oligomers under these conditions.

Immunochemical studies: Antiserum against 64DP reacted with purified 64DP and human sera to form a single precipitation band, but did not react with purified human complement C3 or C3DP in Ouchterlony double diffusion studies(Fig.1-a). Antiserum against C3DP did not react with 64DP(Fig.1-a). 64DP thus appears to be a different protein from C3DP, as judged from molecular weight and immunochemical studies. Although 2 major oncofetal antigens, CEA and α-FP, are used as markers of tumors from specific types, it appears that 64DP is a different protein, since we have been unable to detect CEA(M.Wt. 200,000) or α-FP(M.Wt. 64,000) in the ammonium sulfate fractions in 64DP assay system. Furthermore our preliminary results indicate that the serum 64DP level does not correlate with the amount of these substances.

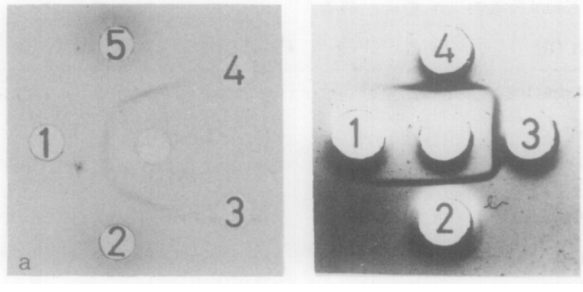

Fig.1. Ouchterlony double diffusion in agar gel.
a) central well: antiserum against 64DP. 1: purified 64DP. 2: serum of normal individual. 3: purified C3DP. 4: purified complement C3. 5: serum of patient with malignant tumor.
b) central well: antiserum against C3DP. 1: purified 64DP. 2: purified complement C3. 3: purified C3DP. 4: serum of normal individual.

Clinical application of 64DP: As shown Fig. 2, thirty sera obtained from normal individuals revealed an average 64DP level of 41.4 μg/ml(range 10-119 μg/ml). In 27 sera obtained from patients with non-malignant diseases, 64DP levels ranged from 19 to 110 μg/ml with the mean value of 49.7 μg/ml. This group included 16 inflammatory diseases, 6 chronic nephritis and 5 collagen diseases. The respective 64DP values were 50 ± 13.7 μg/ml, 27 ± 8 μg/ml and 64 ± 33 μg/ml. Serum 64DP levels for the patients with untreated malignant disease ranged from 110 to 326 μg/ml with the mean value of 175 μg/ml. All of the patients with either stomach or esophageal carcinoma were found to have elevated levels of 64DP, and it was of particular interest to note that there were 8 cases with early stomach cancer(3 adenocarcinomas confined in the mocosa) and 2 cases with early esophageal cancer(2 squamous cell carcinomas not having invaded beyond the submucosa) in this group. It should also be noted that the umbilical cord samples did not show elevated levels of 64DP. These results suggest that serum 64DP determination would have a quite prominent role in diagnosing malignant diseases probably with much wider

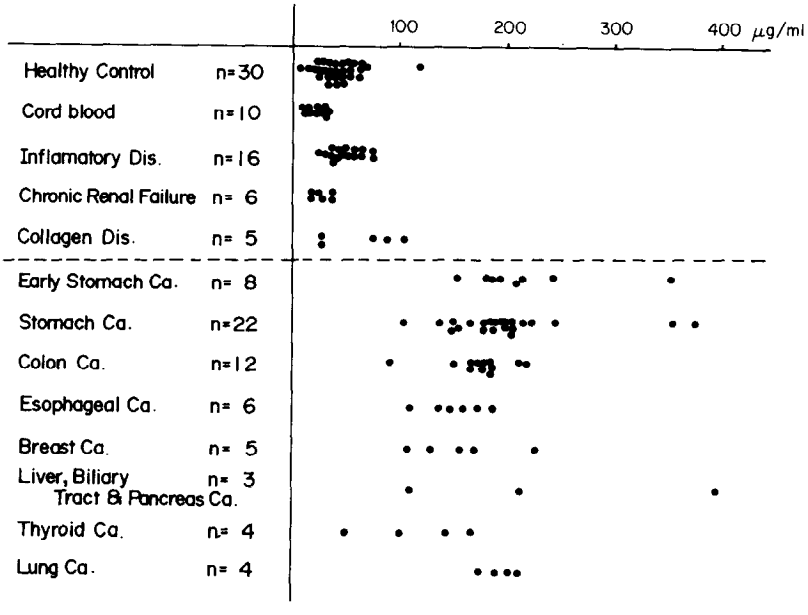

Fig.2. Clinical application of 64DP.

clinical application than C3DP, CEA or α-FP. However, Further studies will be necessary to obtain precise clinical evaluation of serum 64DP levels.

Acknowledgements: This work was supported in part by grant of Tokai medical research grant.

REFERENCES

1. Hoch,S.O., Langmire,R.L. & Hoch,J.A. (1975) Nature 255, 560-562.
2. Parsons,R.G. & Hoch,J.A. (1976) Eur.J.Biochem. 71, 1-8.
3. Parsons,R.G., Longmire,R.L., Hoch,S.O. & Hoch,J.A. (1977) Cancer Research 37, 692-695.
4. Schroder,C., Weinblum,D., Zahn,R.K. & Dose,K. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 473-476.
5. Laemmli,U.K. (1970) Nature 227, 680-685.
6. Fairbanks,G., Stech,T.L. & Wallach,D.F.N. (1971) Biochemistry 10, 2606-2617.