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Purification of Ornithine Decarboxylase-inducing Factor from Cell-free Ascites Fluid of Ehrlich Ascites Tumor and Its Characteristics

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The ornithine decarboxylase-inducing factor (ODC factor) was purified about 1,000-fold in 42% yield from the ascites fluids of an Ehrlich ascites tumor by a combination of centrifugation and concanavalin A (ConA) treatment. A single ip injection of 0.5 μg of the purified factor per mouse resulted in half-maximum induction of liver ODC. The factor was found to be a trypsin- and chymotrypsin-resistant, acidic glycoprotein (pI about 4.43) with a minimum molecular weight of about 70 kilodaltons, containing a disulfide bond(s) in its functional domain. It did not react with ConA. This factor induced retrodifferentiation of liver function, causing a marked increase of prototype M_2 isozyme of pyruvate kinase. It reduced liver catalase activity, and also modified thyroid hormone metabolism, reducing the serum levels of T_4 and T_3. These results suggest that the ODC factor is multifunctional and induces many of the changes observed in a tumor-bearing host.

Key words: ODC factor — Pyruvate kinase isozyme — Thyroid hormone — Cancer cachexia — Ehrlich ascites tumor

Cancer cachexia can not readily be explained as a result of obstruction, competition for a limited supply of nutrients, or anorexia.[1-4] Therefore, we[5-7] have investigated the mechanisms of development of cancer cachexia, assuming that one of its main causes is integrated metabolic abnormalities induced by factors derived from tumors, and that the resulting metabolic derangements may be catastrophic, leading to imbalanced homeostasis. We[8] found a metabolically active factor, named ornithine decarboxylase (ODC[2])-inducing factor or ODC factor, in Ehrlich ascites tumor extracts, and in cell-free ascites fluid. Injection of ODC-factor into normal mice resulted in similar induction of ODC in the liver to that observed in mice bearing Ehrlich ascites tumor. We[9-11] have also investigated various properties of the ODC factor in partially purified preparations. ODC is the first and rate-limiting enzyme in polyamine synthesis in animal tissues[12] and its activity is rapidly induced by various growth stimuli, due to its rapid turnover.[13-16] Polyamines have been shown to stimulate replication, transcription and translation processes, and may regulate cellular metabolism, proliferation and differentiation.[13-16] Therefore, the highly induced activity of liver ODC observed in animals with Ehrlich ascites tumor should cause metabolic deviation, which may be partly mediated by chronic stimulation by the ODC factor.

Thus, for understanding the mechanisms of metabolic deviation of tumor-bearing hosts at the molecular level, it is important to purify the factor, and to determine its biochemical and physiological properties.

In this work, we obtained highly purified ODC factor from cell-free ascites fluid of Ehrlich ascites tumor by a combination of centrifugation and concanavalin A (ConA) treatment, and examined its biochemical and physiological properties. The results obtained suggest that the ODC factor is multifunctional, inducing metabolic deviations and many of the changes observed in Ehrlich ascites tumor-bearing hosts.

On the basis of the present results and other evidence, the mechanisms of development of cancer cachexia are discussed.

MATERIALS AND METHODS

Chemicals DL-[1-^{14}C]Ornithine hydrochloride (40–60 mCi/mmol) was purchased from RCC Amersham, England. [^{125}I]T_4 (SA 1.2 mCi/μg) and [^{125}I]T_3 (SA 1.2 mCi/μg) were obtained from New England Nuclear Corp. (NEN, Boston, MA). Crystalline trypsin (Miles Labs.), trypsin inhibitor (Sigma), chymotrypsin (Type V, Sigma), pronase (Calbiochem-Hoechst), neuramidase, mixed glycosidase (Miles Labs.), containing twelve kinds of glycosidases (α- and β-mannosidases, α- and β-glycosidases, α- and β-galactosidases, α-L-fucosidase, β-

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[2] Abbreviations: ODC factor, ornithine decarboxylase-inducing factor; PK, pyruvate kinase; LDH, L-lactate dehydrogenase; 5′-DI, 5′-deiodinase; ConA, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TPA, 12-O-teradecanoylphorbol 13-acetate; ip, intraperitoneal or intraperitoneally.

xylosidase, α- and β-N-acetylglucosamidases, and α- and β-N-acetylgalactosamidases), which was purified from *Turbo cornutus*, and α-methyl-α-D-mannoside were from Nakarai Tesque (Kyoto), α-1-antitrypsin from human plasma and T_4 and T_3 for use as standards were from Sigma Chemical Co. (St Louis, MO), and RNA polymerase B for molecular weight markers was from Seikagaku Kogyo (Tokyo). DE-52 cellulose was obtained from Whatman. Antisera against T_4 and T_3 were obtained from Miles Inc. (Kankakee, IL). All other chemicals were of the highest grade commercially available.

Clone F2, obtained by Dr. C. Kahana (Weizmann Institute of Science, Israel),[17] which contains a 1.1 kb insert of mouse kidney ornithine decarboxylase-cDNA in pBR 322, was kindly provided by Prof. S. Hayashi (Jikei University School of Medicine) with permission from Dr. C. Kahana.

Animals and treatments Male ICR mice weighing 25–35g were provided by Japan Charles River Co. (Atsugi). Ehrlich ascites tumors were maintained in the peritoneal cavity of mice by transfer every ten days. Ascites fluid was collected 10 days after inoculation of tumor cells, centrifuged at 9,000g for 10 min and kept at -20°C until use. This cell-free ascites fluid was used as starting material for purification of ODC factor.

The ODC factor, and control saline were injected intraperitoneally (ip). The animals were killed by decapitation, and their livers (for ODC assay) were quickly excised and washed with cold saline. The livers were then homogenized in 4 volumes of 0.25 M sucrose containing 1 mM dithiothreitol (DTT) in a Dounce homogenizer. The homogenates were centrifuged at 10^5g for 50 min and the supernatants were used for enzyme assay. Tissue extracts for assay of pyruvate kinase (PK) were obtained as follows. Livers were homogenized in 3 volumes (w/v) of 50 mM potassium phosphate buffer, pH 6.5, containing 0.1 M KCl, 5 mM $MgCl_2$ and 1 mM EDTA in a Dounce homogenizer. The homogenates were centrifuged at 10^5g for 1 h and the resultant supernatants were used for assay of PK activity.

For determination of the effect of ODC factor on thyroid hormone metabolism in mice, 10 μg of highly purified ODC factor per mouse per day was injected ip for 4 or 8 days. Control animals were received ip injections of saline instead of ODC factor. The mice were killed by decapitation on day 5 or 9, their blood was collected and their serum was separated and stored at -20°C until used for assays of T_4 and T_3. The livers of these mice were also rapidly removed, washed with ice-cold saline, frozen in dry ice, and stored at -80°C until used for assay of 5′-deiodinase (5′-DI) activity.

Definition of one unit of activity of the factor responsible for increasing liver ODC activity In general, it is complicated to determine exactly the biological activities of various substances by bioassay. In this study, one unit of activity was tentatively defined as follows: when 1 mg of the factor protein causes half-maximal induction of liver ODC, its factor activity was defined as one unit. Therefore, the reciprocal value of quantity (mg) of the factor protein responsible for half-maximal induction of liver ODC activity, which was estimated from the dose-response curve of liver ODC induction, was defined as specific activity. Unless otherwise stated, liver ODC activities of control animals, which were injected with saline instead of ODC factor or other substances, are not indicated because the values were always in the range of 0–4 pmol CO_2/h/mg protein.

Enzyme assay ODC activity was determined by measuring liberation of CO_2 from DL-[1-^{14}C]ornithine by the method of Russel and Snyder[18] with a slight modification.[11] The assay mixture contained 40 mM Tris-HCl buffer, pH 7.4, 0.2 mM pyridoxal-5-phosphate, 1 mM DTT, 0.4 mM L-ornithine containing 0.25 μCi of radioactivity, and 0.5 ml of adequately diluted extract in a final volume of 1.25 ml. Unless otherwise stated, the activities of ODC were assayed at 37°C in the supernatants of tissue homogenates obtained 20 h after injection of the ODC factor or control saline.

PK activities were assayed at 30°C by the NADH-LDH coupling method described previously.[19, 20] The PK isozymes L and M_2 in extracts from tissues treated with ODC factor or saline were assayed differentially in the presence and absence of M_2-IgG,[20] and the following equations were employed: (total activity) = (L type activity) + (M_2 type activity). (L-Type activity) = (total activity in the presence of M_2-IgG). Therefore, (M_2-type activity) = (total activity) − (total activity in the presence of M_2-IgG, or L-type activity).

5′-DI catalyzes the conversion of T_4 to T_3. In this study the 5′-DI activity of the microsomal fraction of liver cells was assayed by the method of Nauman *et al.*[21] with a minor modification. The T_3 generated and remaining T_4 were assayed by radioimmunoassay (RIA) as described below. The reaction mixture contained 50 mM Tris-HCl buffer (pH 6.0), 3 mM EDTA, 5 mM DTT and 500 μg of microsomal protein in a final volume of 0.4 ml. The reaction was started by adding 1.2 μM L-T_4 solution and was carried out with shaking for 60 min at 37°C. The reaction was stopped by adding 0.8 ml of ice-cold 99.5% ethanol and placing the mixture in an ice bath. The 5′-DI activity was expressed as newly generated T_3 in pmol/mg protein/min. Reaction mixture without T_4 was used to measure the T_3 generated from endogenous substrate (blank control). In addition, the microsomal fraction was added to reaction mixture containing 99.5% ethanol (0 time control) for measurement of endogenous T_3 or T_3 present in the substrate.

Assay of thyroid hormones T_4 and T_3 T_4 and T_3 concentrations were measured by RIA by the method of Mashita *et al.*,[22] which is a modification of the method of Lieblich and Utiger.[23] Antisera against T_4 and T_3 were diluted 2,000 times in the reaction mixture for T_4 determination and 6,000 times in that for T_3 determination as described in the manufacturer's protocol. Radioactive T_4 and T_3 were used at final levels of 10,000 cpm/tube. Radioactivities were counted in a γ-counter (Model MINAXI autogamma 5000 series, Packard Japan Co., Tokyo).

Poly(A^+)-RNA preparation and ODC mRNA content The ODC-cDNA insert was purified by gel electrophoresis on 4% polyacrylamide gel after cleavage of the plasmid pBR322 containing mouse kidney ODC-cDNA (clone F2) with PstI and electroelution on DE-82 ion exchange paper. The cDNA probe was labeled with [α-^{32}P]dATP with a Multiprime DNA-labeling system (Amersham Corp.). The labeled cDNA probe was purified with phenol/chloroform and then by centrifugation through a G-50 spun column.[24] Total liver RNAs from the livers of control mice treated with saline and test mice treated with the highly purified factor were isolated by the guanidine thiocyanate-LiCl method.[25] Poly(A^+)-RNA was purified from total RNA on an oligo(dT) cellulose column.[24] The content of ODC mRNA was determined by Northern blot analysis by hybridization with the mouse ODC cDNA probe prepared as described above.

FPLC chromatofocusing The DE-52 fraction of the ODC factor preparation was subjected to chromatofocusing in a Mono P HR 5/20 column connected to a Pharmacia FPLC system (Pharmacia Fine Chemicals AB, Uppsala). The buffer systems used were as follows: starting buffer, 0.025 *M* bis-Tris buffer adjusted to pH 7.1 with iminodiacetic acid (IDA); elution buffer, Polybuffer (Pharmacia Fine Chemicals AB) adjusted to pH 4.0 with IDA. Fractions of 1 ml were collected at a flow rate of 1.0 ml/min.

Determination of molecular weight The molecular weight of the highly purified ODC factor was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). RNA polymerase B subunits were used as molecular weight markers.

Protein determination Protein concentration was determined by the method of Lowry *et al.*[26] or with a Bio-Rad protein assay kit.

RESULTS

Purification of the ODC factor from cell-free ascites fluid Cell-free ascites fluid that had been stored at $-20°C$ (see "Materials and Methods") was used as the starting material. The purification procedure described below is simpler and faster than previous methods[10] because, as ODC-factor molecules are present as aggregates in ascites, a centrifugal procedure was used as the first step.

Step 1: First centrifugation The action of contaminating protease on the factor protein was prevented by adding 1 m*M* phenylmethylsulfonyl fluoride (PMSF) to the cell-free ascites fluid. This ascites fluid was then adjusted to pH 7.5 and centrifuged at 9,000*g* for 10 min to remove insoluble materials. This supernatant was used as original ascites fluid for purification of ODC factor. The supernatant (140 ml which contained 6535 mg of protein) was recentrifuged at 10^5g for 2 h. The resultant supernatant was named Sup-1. The precipitate was suspended in 10 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* PMSF and homogenized in a Dounce homogenizer, and the homogenate was centrifuged at 9,000*g* for 10 min to remove insoluble materials. The resultant supernatant was named Ppt-1, which contained 58.8 mg of protein. Fig. 1 shows the dose-responses of ODC induction by the cell-free ascites fluid (panel A), Sup-1 (panel A) and Ppt-1 (panel B). As demonstrated by ODC activity, most of the ODC-factor activity in the ascites fluid was clearly recovered in Ppt-1 (panel B) with scarcely any in Sup-1 (panel A). From these dose-response curves the amounts of the original cell-free ascites fluid and Ppt-1 required for half-maximal induction of ODC in the liver were estimated as about 480 μg/mouse and 5.6 μg/mouse, respectively, from which their specific activities were calculated to be 2.1 U/mg protein and 178.6 U/mg protein. Thus, recovery of the ODC-factor activity was 76.5% with an 85-fold increase in specific activity over that in ascites fluid. These results indicate that ultracentrifugation is a very effective method for purification of the factor.

Second centrifugation To eliminate contaminating fibrinogen, 20 m*M* $CaCl_2$ was added to Ppt-1, and the Ppt-1 solution was incubated in a water-bath at 30°C with shaking for 20 min. Insoluble material was removed by centrifugation at 9,000*g* for 10 min and the supernatant was recentrifuged at 10^5g for 2 h. The resultant supernatant was named Sup-2. The precipitate was homogenized in Tris-HCl buffer and centrifuged as described above and the resultant solution was named Ppt-2. Almost all the ODC-factor activity was recovered in the 10^5g precipitate, or Ppt-2.

Step 2: DE-52 cellulose column chromatography Ppt-2 was subjected to stepwise chromatography on a column of DE-52 cellulose equilibrated with 20 m*M* sodium phosphate buffer, pH 7.65. About 54 mg of the Ppt-2 fraction was applied to the column, and the column was washed with the equilibration buffer until the absorbance of the eluate at 280 nm returned to the base line. Material was then eluted stepwise with the same buffer containing 50 and 125 m*M* KCl, respectively. Fig. 2 shows the

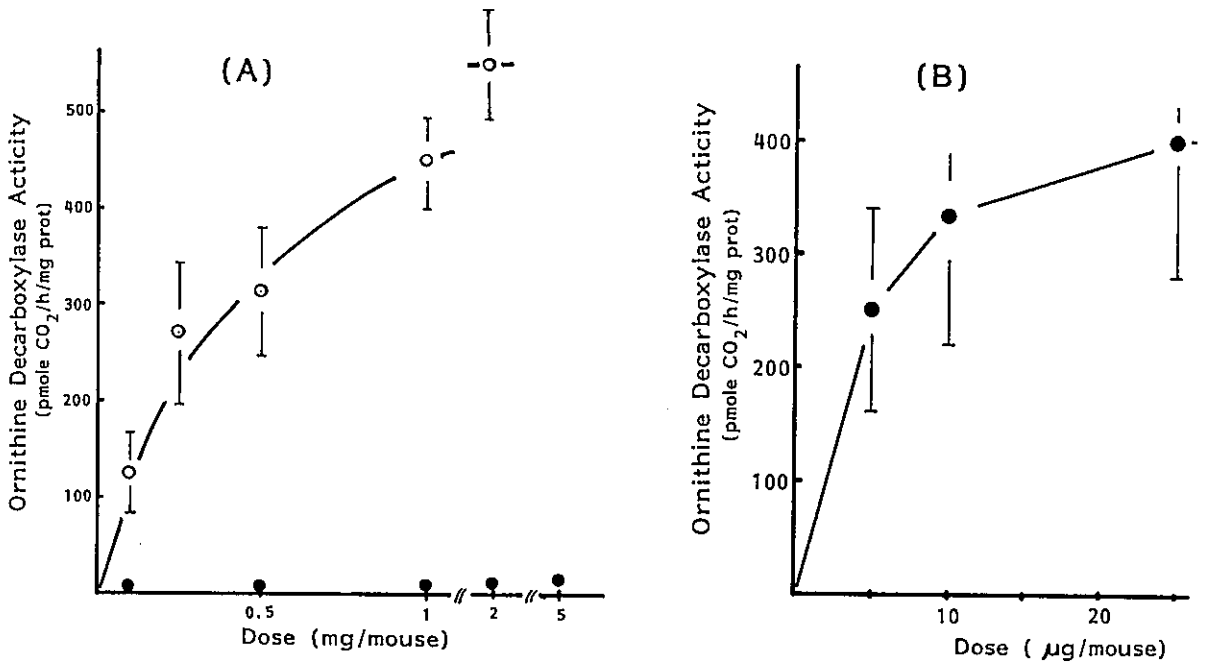

Fig. 1. Dose-response relation for ODC induction by the original cell-free ascites fluid (panel A), Sup-1 (panel A) and Ppt-1 (panel B). The indicated concentrations of the factor preparations were injected ip into mice. The mice were killed 20 h later and their liver OCD activity was measured. In panel A, (○) and (●) show the enzyme activities induced by the original cell-free ascites fluid and Sup-1, respectively. In panel B, (●) indicates the enzyme activity induced by Ppt-1. Values are means ±SD for four mice.

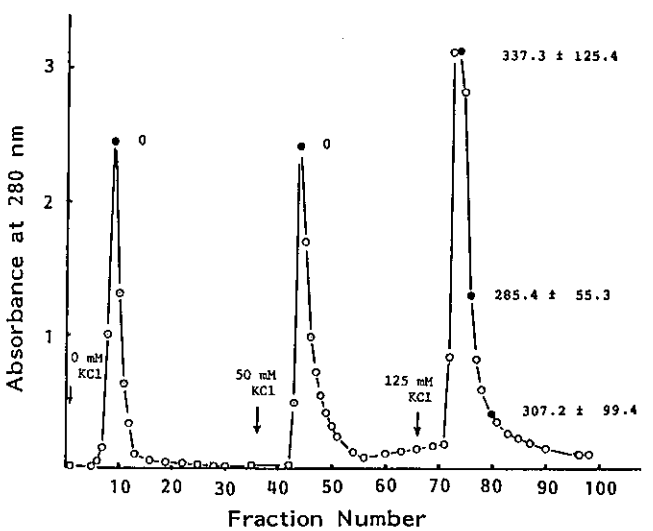

Fig. 2. Elution profile of the Ppt-1 fraction from a DE-52 cellulose column by stepwise increase in KCl concentration. About 54 mg of Ppt-1 preparation was applied to a DE-52 cellulose column (1.2×9 cm) equilibrated with 20 m*M* sodium phosphate buffer, pH 7.65. Material was eluted stepwise with increasing KCl concentrations in the same buffer as indicated by arrows. Samples of 20 μg protein of each fraction were injected ip into mice. The mice were killed 20 h later for measurement of liver ODC activity. (○) and (●) indicate the absorbance at 280 nm and samples used to measure ODC activity, respectively. Values for enzyme activity (pmol CO_2/ mg protein/h) are means±SD for four mice.

elution profile of the activity of the ODC factor using this procedure. Activity was indicated as liver ODC activity induced when each fraction was administered at the dose of 20 μg protein/mouse. Activity was all recovered in peak 3, eluted with 125 m*M* KCl. Fractions in peak 3 were combined, concentrated with a Diaflow YM 5 ultrafiltration membrane, and centrifuged at 9,000*g* for 10 min to remove insoluble materials. The yield of the protein was 14.7 mg. The amount of the factor required for half-maximal induction of hepatic ODC activity was about 1.8 μg, which corresponds to a specific activity of 555.6 U/mg protein.

Step 3: ConA Sepharose chromatography About 14.6 mg of the factor from Step 2 (DE-52 fraction) was applied to

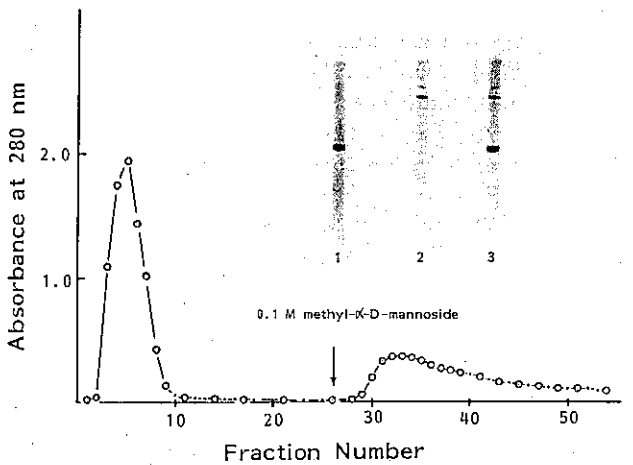

Fig. 3. Concanavalin A Sepharose chromatography of the fraction from DE-52 cellulose. The DE-52 fraction (14.6 mg) was applied to a ConA Sepharose column (1.2×3.5 cm) equilibrated with 0.15 *M* NaCl in 25 m*M* Tris-HCl buffer, pH 7.5. The column was washed with the same buffer to remove unadsorbed material, and then adsorbed material was eluted with the same buffer containing 0.1 *M* methyl-α-D-mannoside as indicated by the arrow. (○), absorbance at 280 nm. The insert shows the SDS-PAGE profiles of the unadsorbed (lane 1) and adsorbed (lane 2) fractions and the DE-52 fraction applied to the ConA Sepharose column (lane 3).

a ConA Sepharose column equilibrated with 0.15 *M* NaCl in 25 m*M* Tris-HCl buffer, pH 7.5. The column was washed with the same solution until the absorbance of the eluate at 280 nm returned to the base line, indicating removal of unabsorbed protein. The adsorbed protein was eluted with the same NaCl solution containing 0.1 *M* methyl-α-D-mannoside (Fig. 3). The unadsorbed and adsorbed fractions, respectively, were pooled and concentrated in a mini-ultrafiltration system (Molcut, Amicon). Formed insoluble materials were removed by centrifugation. The insert of Fig. 3 shows the SDS-PAGE profiles obtained by Laemmli's method[27] of the pooled unadsorbed, and adsorbed fractions and the DE-52 fraction applied to the ConA Sepharose column; the unadsorbed fraction gave a rather broad, single band on SDS-PAGE. Densitometry of the stained gel after SDS-PAGE of the unadsorbed fraction also showed a single band. Activity of the ODC factor was detected only in the unadsorbed fraction from the ConA Sepharose column. Fig. 4 shows the dose-dependence of the effect of the unadsorbed fraction in ODC induction: a single injection of 0.5 μg/mouse of this unadsorbed fraction caused half-maximal induction of ODC activity in the liver of normal mice. The specific activity of the highly

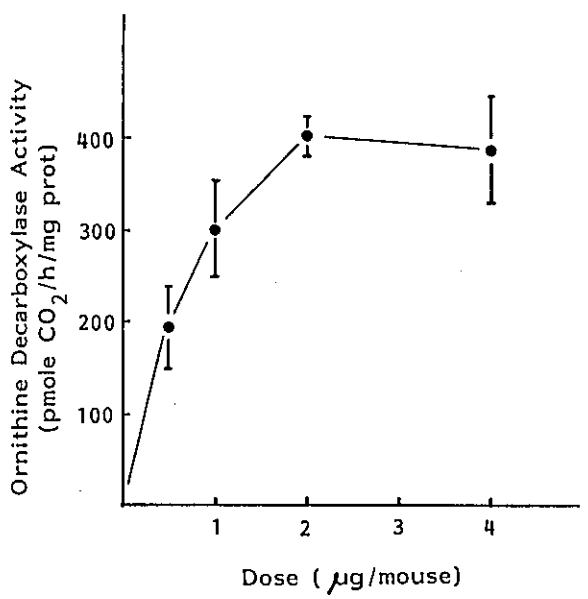

Fig. 4. Dose-dependence of ODC induction by the fraction not adsorbed on ConA (highly purified preparation). Various concentrations of the highly purified preparation were injected into mice ip. The animals were killed 20 h later, and their liver ODC activities were measured. Values are means±SD for four mice.

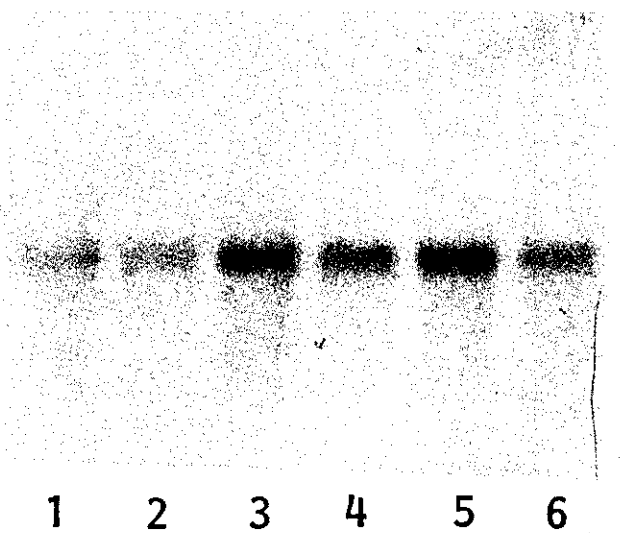

Fig. 5. ODC-mRNA levels in the livers of mice treated with the highly purified preparation. Total tissue RNA was isolated from the liver 0 (lane 1), 14 (lane 2), 16 (lane 3), 18 (lane 4), 20 (lane 5) or 22 (lane 6) h after administration of 10 μg of highly purified factor per mouse, and poly(A^+)-RNA was separated from total RNA on an oligo (dT) cellulose column. Samples (2 μg of poly(A^+)-RNA) were subjected to electrophoresis in agarose gel (0.8%) containing formaldehyde, blotted onto a cellulose membrane, and hybridized with a mouse kidney ODC-cDNA probe.

purified factor preparation was calculated to be 2,000 U/mg protein. About 2.91 mg of protein was obtained. Thus, the overall recovery of the ODC factor was 42.4% with about 1,000-fold increase in specific activity over that in ascites fluid.

Northern blot hybridization analysis of poly(A^+)-RNA Fig. 5 shows the results of Northern blot hybridization analysis of poly(A^+)-RNA from the livers of normal mice and mice treated with purified ODC factor using a mouse kidney ODC-cDNA probe. These results indicate enhanced expression of ODC-mRNA in the livers of mice treated with the ODC factor. The time course of increase in ODC-mRNA was also comparable to that of increase in ODC activity reported previously.[11]

Characterization of the ODC factor Molecular weight: The minimum molecular weight of the highly purified ODC factor was estimated as 70 kd by SDS-PAGE with RNA polymerase B subunits as molecular weight markers (Fig. 6).

Apparent isoelectric point: The DE-52 fraction was subjected to Mono P chromatofocusing in a gradient of pH 4–7. The ODC-factor activity was recovered in the pI 4.43 fraction (data not shown).

Effects of various enzymes and chemical treatments on the ODC factor: Table I (A, B, C, D and E) shows the effects of various enzymes and chemical treatments on the highly purified factor preparation. In F in the same table, liver ODC activities of control, and Ehrlich ascites tumor-bearing animals 7 days after tumor inoculation are given for comparison. The activity of the factor in the highly purified preparation was markedly reduced by treatment with pronase (B) and mixed glycosidase (C), but not with neuramidase (D). It was also markedly

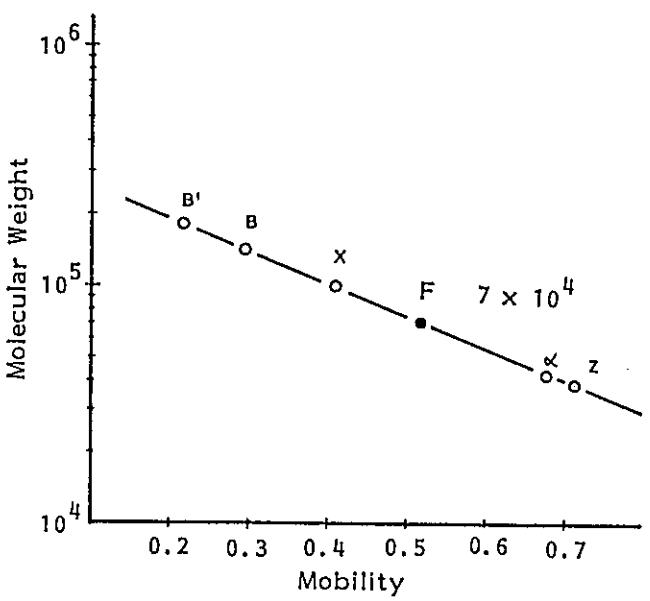

Fig. 6. Determination of the molecular weight of the highly purified factor preparation by SDS gel electrophoresis. The molecular weight markers used were: RNA polymerase B subunits; B′ (18×10^4), B (15×10^4); X (10×10^4); α (4.2×10^4); and Z (3.9×10^4). From the results, the molecular weight of the highly purified factor (F) was estimated as 7×10^4 (70K) daltons.

Table I. Effect of Digestive Enzymes and Chemicals on Activity of the Highly Purified Factor

	ODC activity (pmol CO_2/h/mg protein)
A	
Factor (F) alone	224.85±73.2
F+trypsin	263.63±65.03
B	
F alone	261.6±111.68
F+chymotrypsin	222.53±54.59
F+pronase	13.13±3.15[a]
C	
F alone	250.0±100.5
F+mixed glycosidase	17.8±11.6[b]
Mixed glycosidase alone	3.5±0.85
D	
F alone	390.32±141.5
F+neuramidase	419.41±154.85
Neuramidase alone	15.95±9.38
E	
F alone	281.3±167.7
F+DTT	7.1±4.2[c]
F	
Saline control	3.3±0.33
EAT-bearers	340.5±68.45[d]

Samples of 50 μg of the factor in 1 ml of reaction mixture were incubated with the indicated enzymes or chemicals at 30°C. Incubation conditions: A and B, ±50 μg of trypsin or chymotrypsin or pronase for 2 h in 0.1 *M* phosphate buffer (pH 8); C, ±0.5 mg of mixed glycosidase in 0.1 *M* sodium phosphate buffer (pH 6) containing 2 mg of BSA, which was added to protect the factor from protease contaminating the mixed glycosidase; D, ±50 μg of neuramidase in 0.1 *M* sodium phosphate buffer (pH 6); E, ±65 m*M* DTT in 0.1 *M* sodium phosphate buffer (pH 8) for 1 h. Samples of 0.2 ml of factor (corresponding to 10 μg) after each treatment were injected ip into mice. Saline control and Ehrlich ascites tumor (EAT)-bearers indicate saline-injected mice and EAT-bearing ones 7 days after tumor inoculation, respectively. Values are means± SD for four animals. Significance of differences from the value with the factor alone in each group: *a*), $P<0.001$; *b*), $P<0.01$; *c*), $P<0.05$. Significance of difference from saline control: *d*), $P<0.001$.

Table II. Changes in PK Isozymes in the Livers of Mice Treated with Highly Purified Factor

	Pyruvate kinase activity (U/mg protein)		
	Total	L-Type	M_2-Type
Control	0.408±0.038	0.378±0.047	0.030±0.012
ODC factor-injected	0.447±0.040	0.361±0.047	0.086±0.012[a]
EAT-bears	0.451±0.027[b]	0.324±0.011[c]	0.132±0.017[a]

The unadsorbed fraction from ConA was injected ip at 10 μg protein/mouse once a day for three days. The mice were killed next day and their liver PK isozymes were quantified using anti M_2-IgG as described in "Materials and Methods." Values are means±SD for five animals. EAT-bearers indicates Ehrlich ascites tumor-bearing mice. Significant difference from control value: *a*), $P<0.001$; *b*), $P<0.01$; *c*), $P<0.05$.

Table III. Effect of ODC Factor on Thyroid Hormone Metabolism

	Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
Control	8.47±0.44	1.66±0.10	235.61±11.78
EAT bearers	2.67±0.31[a]	0.53±0.06[a]	90.26±9.23[a]
Control (4 days)	9.16±0.5	1.06±0.06	245.90±31.33
Factor (4 days)	6.74±0.47[a]	0.78±0.08[b]	378.85±33.34[b]
Control (8 days)	9.01±0.21	0.96±0.06	278.70±31.85
Factor (8 days)	6.88±0.27[a]	0.78±0.04[c]	661.12±105.04[a]

Experimental procedures were as described in "Materials and Methods." EAT bearers, Ehrlich ascites tumor-bearing mice 10 days after ip inoculation of tumor cells. Data are means±SD for four animals. Significance of differences from respective control value: *a*), $P<0.001$; *b*), $P<0.01$; *c*), $P<0.05$.

reduced by treatment with the S-S reducing reagent DTT (E). These results are consistent with previous findings.[10] In addition we found that the factor is resistant to the proteolytic enzymes trypsin and chymotrypsin (A and B).

Recently, injection of antitrypsin-like protein from Ehrlich ascites fluid into mice was found to stimulate thymidine incorporation into their hepatocytes and production of thymidine kinase in their liver (unpublished results by Kato *et al.*).[28] Therefore, we examined the effect of the human α-1-antitrypsin on liver ODC activity. However, we did not detect any induction of ODC 4 or 20 h after injection of 20, 50 or 100 μg of protein of human α-1-antitrypsin per mouse (data not shown).

Deviation of isozyme patterns of PK and suppression of catalase in the liver induced by the ODC factor To examine whether the ODC factor influences PK isozyme patterns in the liver like a nonhistone nuclear protein obtained from Ehrlich ascites tumor cells previously reported by us,[29] the highly purified preparation was injected at a dose of 10 μg per day into normal mice for 3 days. Table II shows the alterations in the PK isozyme levels in the livers of mice treated with the factor. The total activity tended to increase and the level of the functional, or differentiated, L-type PK isozyme tended to decrease in the liver relative to the levels in control mice, but the changes were not statistically significant. On the other hand, the level of the prototype, or undifferentiated M_2-type PK isozyme increased significantly to about 3 times that in controls. In the case of Ehrlich ascites tumor-bearing animals, total PK and L-type PK activities were significantly increased and decreased, respectively, even 4 days after tumor inoculation, with a marked increase in M_2-type PK activity. The spleens of the ODC factor-treated mice also showed a tendency for hypertrophy (control, 152.5±59.7 mg; factor, 216.2±50.7 mg). We also injected the highly purified preparation ip to determine whether it affected liver catalase. About 20% reduction of the liver catalase activity was observed after a single injection of 10 μg protein of the preparation per mouse (data not shown).

Effect of the ODC factor on thyroid hormone metabolism There are reports of low levels of circulating thyroid hormones in hosts with non-thyroidal tumors.[30-33] As shown in Table III, the serum T_4 and T_3 levels and the liver 5′-DI activity in tumor-bearing mice (10 days after tumor inoculation) were all markedly lower than those in control mice. So, we examined whether the ODC factor influenced thyroid hormone metabolism. The serum levels of T_4 and T_3 in mice treated with 10 μg of the

highly purified preparation of ODC factor once a day were significantly lower than those of control mice treated with PBS after 4 days. On the other hand, daily treatment with the ODC factor increased the liver 5′-DI activity significantly by day 4 and markedly by day 8, in contrast to the effect of tumors on this activity.

DISCUSSION

Cancer cachexia is usually associated with malnutrition, resulting from decreased food intake. As with malnutrition, some of the characteristics of cachexia may be overcome by forced feeding programs such as total parenteral nutrition, that is, intravenous feeding, or hyperalimentation by stomach tube. However, cancer cachexia is not correlated in any simple manner with malnutrition,[1-4] because it cannot be completely reversed without elimination of the cancer.[4,34] These observations strongly suggest that tumor-derived factors as well as malnutrition participate in the genesis of cancer cachexia.

Nakahara and Fukuoka[35] first found a physiologically active factor, the so-called toxohormone, in extracts of all the tumors that they studied. Injection of this factor into normal animals caused many of the host changes seen in hosts with neoplasms. Later, Lucke *et al.*[36] and Suda *et al.*[5] demonstrated that some of the metabolic abnormalities of the tumor-bearing hosts might be mediated by tumor-derived factors. Subsequently, there have been many attempts to purify physiologically active factors belonging to the category of toxohormones from tumors.[7,10,37,38] However, these studies have not been successful, and little is yet known about the biochemical properties and molecular mechanisms of the actions of these factors.

In the present study, the ODC factor was highly purified by a simple, rapid procedure using contrifugal fractionation and ConA Sepharose column chromatography. The specific activity of the highly purified factor is 2,000 U/mg protein and the overall recovery of the factor was 42.4% with about 1,000-fold purification of the factor from the original ascites fluid. Northern blot hybridization analysis showed that this ODC induction was due to increase in ODC-mRNA, confirming a previous result obtained using a transcriptional inhibitor.[11] The factor was found to be a trypsin- and chymotrypsin-resistant, acidic glycoprotein (pI 4.43) with a minimum molecular weight of about 70 kd, containing a disulfide bond(s) in its functional domain. It did not react with ConA. However, human α-1-antitrypsin, at least, did not induce any liver ODC activity in mice. Administrations of growth factors such as epidermal growth factor,[39] never growth factor[40] and prolactin[41] to animals are also known to induce liver ODC. The ODC factor also has growth factor-like activity.[9] However, the mechanism by which it induces liver ODC appears to be different from those of growth factors because the time required for maximum induction of liver ODC activity was about 20 h with ODC factor but 4–6 h with growth factors. The mechanism of ODC induction in the liver by ODC factor is also different from that of ODC induction in the skin by the tumor promoter TPA, because the latter depends on protein synthesis but not RNA synthesis.[42]

We also examined various other effects of the ODC factor on metabolism in mice. Continuous administration of the ODC factor (10 μg of the factor per mouse once a day for 3 days) induced an alteration of the isozyme pattern of PK in the liver; that is, a marked increase in prototype, or undifferentiated isozyme (M_2-type). This finding suggests that the factor stimulated the gene expressing the M_2-type in the liver. The M gene is apparently switched on for energy supply associated with cell growth, as observed in fetal tissues, regenerating liver, host liver bearing a cancer, and cancer cells, while it is suppressed in the normal state in adult liver.[7] Thus, increased expression of the M gene induced by the factor appears to represent retrodifferentiation of liver function, as observed in tumor-bearing hosts. On the other hand, the factor did not significantly reduce functional or differentiated isozyme (L-type), unlike Ehrlich ascites tumor. At least two explanations for this result are possible. One is that additional administration may be required for significant reduction of L-PK activity. Another is that some other factors which suppress the gene expressing the L-PK may be present in Ehrlich ascites tumor-bearing hosts, because we recently found that an endogenous factor, IL1, which is expected to be produced and released in excess or inappropriately from host tissues as a consequence of interaction of these host tissues with invaders such as cancer and microorganisms, also induced deviation of PK isozyme patterns in mouse liver, markedly increasing the activity of M_2-PK with a significant decrease in L-PK in the liver.[47] The factor also reduced liver catalase activity. Furthermore, continuous administration of the factor to mice at 10 μg of protein per mouse once a day for 4 or 8 days modified thyroid hormone metabolism, reducing the serum levels of T_4 and T_3. These changes mimic those in tumor-bearing hosts. However, liver 5′-DI activity, which is a marker of peripheral metabolism of thyroid hormones, was greatly increased by the ODC factor in contrast to the effects of Ehrlich ascites tumor, and serum thyroid hormone reducing factor (STRF) derived from Walker 256 carcinosarcoma on this activity.[43] Administration of STRF to rats significantly reduced liver 5′-DI activity. The marked increase of liver 5′-DI activity induced by the ODC factor may result from switching-on of compensation mechanisms of the normal body for reduction

of serum thyroid hormone levels, suggesting that some other factor(s) that suppresses such mechanism(s) is present in tumor-bearing hosts as described above in connection with the deviation of PK isozymes. Many of these changes mimic those in cancer-bearing hosts and also indicate that the ODC factor is multifunctional. It was recently reported that cytokines such as tumor necrosis factor/cachectin and IL1 also induce deviation of thyroid hormone metabolism. These endogenous factors may also be strong candidates as mediators of cancer cachexia.[44-47] Therefore, on the basis of the present results and various lines of evidence we conclude that the combinations of metabolic deviations in tumor-bearing hosts induced by the tumor-derived factor(s), endogenous factors such as cytokines and anorexia may be main causes of development of cancer cachexia leading to imbalanced homeostasis.

Tumor-bearing hosts may be chronically influenced by both tumor-derived factors and endogenous factors. Therefore, studies are required on whether the effects of these two types of factors are additive or synergistic.

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