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Characterization of a 54 kDa, α_1-Antitrypsin-like Protein Isolated from Ascitic Fluid of an Endometrial Cancer Patient

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A protein factor which stimulated [^{3}H]thymidine uptake into free hepatocytes prepared from normal mouse liver was detected in the ascitic fluid of gynecological cancer patients. The factor was subsequently further purified from the ascitic fluid of an endometrial cancer patient by DEAE-Sephacel, Sephadex G-150 and Phenyl-Sepharose CL-4B column chromatographies, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a single protein band of 54,000 Da, designated tentatively as 54K ascitic protein (54K-AP). 54K-AP was similar to human α_1-antitrypsin (α_1-AT) in terms of SDS-PAGE and immunological behavior, but was slightly different in terms of amino acid sequence and isoelectric point. Although 54K-AP inhibited the activities of bovine trypsin and α-chymotrypsin as did human α_1-AT, 54K-AP inhibited the plasminogen activator released from human endometrial cancer Ishikawa cells more efficiently than α_1-AT. Because, in contrast to normal serum, the serum from the endometrial cancer patients stimulated [^{3}H]thymidine uptake into hepatocytes, the possibility arises that 54K-AP could be produced by the cancer host as a defence mechanism against the cancer.

Key words: α_1-Antitrypsin-like protein — Thymidine uptake — Endometrial cancer — Tumor host ascites

In the tumor-bearing animal, a number of metabolic changes have been observed, such as alterations in the metabolism of nucleic acids and their precursor nucleotides, especially in the liver.[1-3] We have found that the activity of cytosolic thymidine kinase (EC 2.7.1.21) (TK)[4] as well as thymidylate synthetase increased transiently in the liver, accompanied with an increase of [^{3}H]thymidine uptake, after the transplantation of Ehrlich ascites tumor into a mouse. A protein factor which stimulated TK activity in the liver *in vivo* was then purified from the cell-free ascitic fluid of the Ehrlich ascites tumor-bearing mouse, based on monitoring of the *in vitro* stimulation of [^{3}H]thymidine uptake into free hepatocytes prepared from a normal mouse liver.[4] Moreover, it was revealed that this factor was similar to α_1-antitrypsin (α_1-AT), a trypsin inhibitor in plasma, in its structure and function.

It is well known that the serum level of α_1-AT is elevated in patients with various kinds of cancer,[5,6] but its biological role in these patients has not yet been elucidated. In the present study, we isolated an α_1-AT-like protein factor from the ascitic fluid of an endometrial cancer patient, again while monitoring the stimulation of the [^{3}H]thymidine uptake into free mouse hepatocytes. Characterization of the purified protein revealed that it was different from α_1-AT obtained from normal human serum.

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[4] The abbreviations used are: TK, thymidine kinase; α_1-AT, α_1-antitrypsin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; BSA, bovine serum albumin; PA, plasminogen activator; HBS, Hanks' buffered saline; AF, ascites factor; 54K-AP, 54 K ascitic protein.

MATERIALS AND METHODS

Materials [Methyl-^{3}H]Thymidine (43.0 Ci/mmol) was purchased from Amersham Corp., Buckinghamshire, England. Filter papers (Toyo No. 514) were supplied by Toyo Roshi, Ltd., Tokyo. DEAE-Sephacel, Sephadex G-150, Phenyl-Sepharose CL-4B and all marker proteins that were used for the gel filtration, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Affi-Gel blue was obtained from Bio-Rad Lab., Richmond, Ca. α-Chymotrypsin, α_1-AT (partially purified from human placenta) and anti-human α_1-AT antibody (rabbit IgG fraction) were purchased from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin (BSA; fraction V) was obtained from Armour Pharmaceutical Co., Ill.; trypsin (bovine pancreas) was from Wako Pure Chemical Industries, Ltd., Tokyo; casein was from Koso Chemical Co., Ltd., Tokyo; S-2251 was from Kabi Diagnostica, Stockholm, Sweden, and human plasminogen was from

Green Cross Co., Osaka. All of the other chemicals were of analytical grade.

Collection of effusions Ascitic and pleural effusions were obtained from patients with malignant and benign diseases. In all of the effusions of the former group of patients, cancer cells were observed. Individual ascitic effusions were collected from patients with endometrial cancer (cases #1 and 2), ovarian cancer (cases #3, 4 and 5), mixed mesodermal tumor (case #6) and hepatocellular carcinoma (case #7). The pleural effusion was obtained from a patient with esophageal cancer (case #8). The other ascitic effusions were obtained from patients with benign diseases, i.e., liver cirrhosis (case #9) and endometriosis (case #10). Each effusion was centrifuged at 15,000*g* for 15 min to remove cells and cell debris, and was stored at −40°C until used.

Collection of conditioned medium of human endometrial cancer Ishikawa cells cultured in serum-free medium The Ishikawa cell line had been established in the presence of 15% fetal calf serum from human endometrial cancer[7] and it secretes plasminogen activator (PA) (unpublished data). We cultivated this cell line continuously until it could grow in serum-free Biorich 1 Medium (Flow Laboratories, Sydney, Australia), consisting of a mixture of Dulbecco's minimum essential medium and Ham's F-12 medium (1:1, v/v), and containing no additives such as growth factor, except for some kinds of minerals. Cells (2.5×10^5 in 5 ml of the serum-free medium) were inoculated, and then the conditioned medium in the stationary phase of cell growth (5×10^5 cells per ml) was collected and dialyzed against 10 m*M* Tris-HCl buffer (pH 7.4) for use in the experiment.

Assay for [^{3}H]thymidine uptake into free hepatocytes isolated from mouse The assay was performed by a modified version of the method of Morley and Kingdon.[8] The liver from a male mouse (ddY strain) was crushed through a stainless-steel sieve and suspended in Hanks' buffered saline (HBS), followed by centrifugation at 15*g* for 5 min. The obtained cell pellet was resuspended in 9 volumes of the same solution. An aliquot (10 μl) of the cell suspension was incubated with 90 μl of the sample for 15 min at 37°C, and then 1 μCi of [methyl-^{3}H]thymidine was added. After incubation for another 15 min at 37°C, the mixture was applied to a 4.2 cm^2 disc of filter paper (Toyo No. 514), which was subsequently washed twice with ice-cold 5% TCA, once with ethanol, and finally once with ethyl ether. The radioactivity (S) on the dried disc was measured in 7 ml of a toluene-based scintillator cocktail (containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)benzene/liter of toluene) with an Aloka LSC-703 liquid scintillation spectrometer. Basal [^{3}H]thymidine uptake (C) into free hepatocytes as a control was determined by the incubation of the hepatocytes with HBS instead of the sample, and then the stimulation of thymidine uptake was calculated by using the following formula: (S−C)/C, and expressed in units.

Trypsin or α-chymotrypsin inhibition assay Trypsin activity was determined as described by Laskowski.[9] Trypsin- or α-chymotrypsin-inhibitory activity was measured by adding the sample (inhibitor) to the assay mixture for determination of trypsin activity. The assay mixture contained 0.44% casein, bovine trypsin or bovine α-chymotrypsin, 10 m*M* Tris-HCl buffer (pH 8.0), 5 m*M* $CaCl_2$, and the sample in a final volume of 0.45 ml. After incubation for 30 min at 37°C, 5% TCA was added, and then the absorbance of the supernatant of the mixture was measured at 280 nm by spectrophotometry. When the value of the control, measured with the same buffer as above instead of the sample, was defined as 100%, the inhibitory activity of the sample was expressed in terms of the enzyme activity measured with the sample as percent of the control, that is, the remaining activity of the enzyme.

PA-inhibition assay PA activity was measured by a modified version of the method of Shimada *et al.*[10] PA-inhibitory activity was determined by adding the sample (inhibitor) to the assay mixture for PA activity. Briefly, 0.73 m*M* chromogenic substrate S-2251 (H-D-Val-Leu-Lys-pNA), 10 m*M* Tris-HCl buffer (pH 7.4), an aliquot of conditioned medium of human endometrial cancer Ishikawa cells containing PA activity, and the sample were incubated for 30 min at 37°C with human plasminogen at a final concentration of 0.5 CU/ml. After the addition of 50% acetic acid, the absorbance of the supernatant of the mixture was measured at 405 nm by spectrophotometry. The absorbance of the control, measured with the same buffer as above instead of the sample, was defined as 100%, and the inhibitory activity of the sample was expressed as percent of the control, from the remaining PA activity measured with the sample.

Electrophoresis SDS-PAGE of the purified protein was performed on a slab gel plate made of 12% polyacrylamide containing 0.2% SDS as described by Laemmli.[11] Electrophoresis was performed at 30 mA, and the gel was stained for protein by using Coomassie Brilliant Blue R-250. For the molecular weight determination, BSA (67,000), ovalbumin (43,000) and chymotrypsinogen A (25,000) were used as marker proteins. IEF was carried out at 15°C and 500 Vh on the gel plate (PhastGel IEF 4-6.5, Pharmacia) with an electrophoretic apparatus (Phast System, Pharmacia). The gel was stained at 50°C by using "PhastGel Blue R" solution (Pharmacia). The isoelectric pH values were determined by using marker proteins (IEF pI calibration kit, Pharmacia), including soybean trypsin inhibitor (pH 4.55), β-lactoglobulin A (pH 5.20) and bovine carbonic anhydrase (pH 5.85).

Double-immunodiffusion on agarose gel plate This was performed according to the method of Ouchterlony.[12]

Amino acid sequencing Determination of the sequence of 15 amino acids from the amino terminal of the purified protein was performed by Edman degradation with an automatic gas phase sequencer (Model 477A, Applied Biosystems Inc.) and identification of each released amino acid by reversed-phase HPLC (Model 120A, fitted with a PTH-C18 column 2.1 mm in diameter).

Protein determination The amount of protein was determined by the method of Lowry *et al.*[13] using BSA as a standard.

RESULTS

Stimulation of [^{3}H]thymidine uptake into isolated murine hepatocytes by ammonium sulfate fraction of effusions from patients with malignant and benign diseases We found that the ammonium sulfate fraction (AS:40-70) of human effusion, which was solubilized in 40% saturated ammonium sulfate solution and then precipitated by adding solid ammonium sulfate so as to reach 70% saturation, stimulated [^{3}H]thymidine uptake of free hepatocytes. As shown in Fig. 1, the average ($\pm$SD) stimulatory activity in the effusions derived from gynecological cancer patients (cases #1, 2, 3, 4, 5 and 6) was 0.52 ± 0.09 units/mg protein, which was higher ($P < 0.01$; Student's two-tailed t test) than those (0.28 ± 0.04) from non-gynecological cancers (cases #7 and 8) including benign diseases (cases #9 and 10).

Purification of 54K ascitic protein from the effusion of an endometrial cancer patient A factor which was assumed to stimulate [^{3}H]thymidine uptake was purified, guided by monitoring of the stimulation of [^{3}H]thymidine uptake into free hepatocytes, from the ascitic fluid of endometrial cancer case #1, which showed the highest stimulatory activity (0.67 units/mg protein) in Fig. 1. Purification was performed by a slight modification of

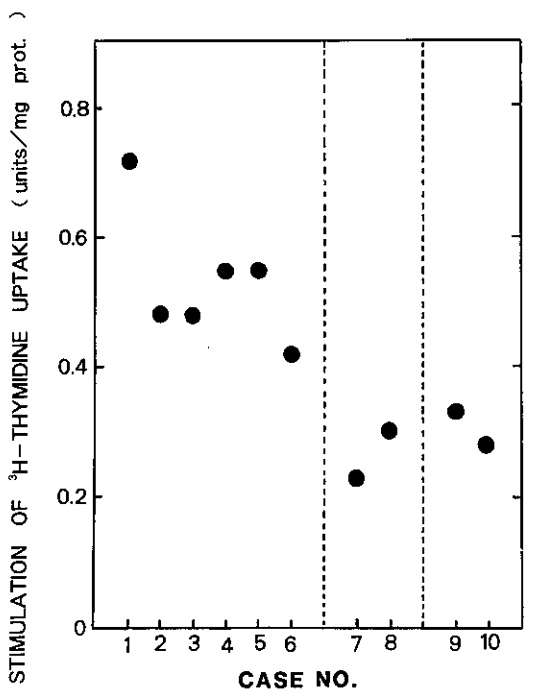

Fig. 1. Stimulation of [^{3}H]thymidine uptake into free hepatocytes by ammonium sulfate fractions of the effusions from patients with malignant and benign diseases. Each patient is identified in "Materials and Methods" by case number. AS: 40-70 fractions of the effusions were prepared as described in the text. [^{3}H]Thymidine uptake was measured as described in "Materials and Methods," and its stimulation was expressed as units/mg protein of the AS:40-70.

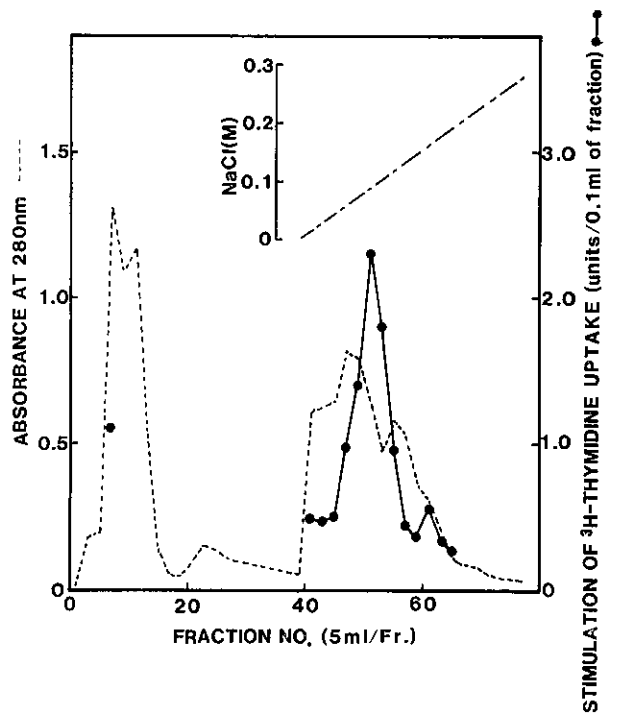

Fig. 2. DEAE-Sephacel column chromatography of the ascitic fluid from an endometrial cancer patient. About 50 ml of the ascitic fluid dialyzed against 10 mM phosphate buffer (pH 7.0) was applied to a DEAE-Sephacel column. Chromatography was performed as described in the text. Every 5 ml of the eluate was collected in a fraction, and stimulatory activity for [^{3}H]thymidine uptake into free hepatocytes of 0.1 ml of each fraction (●) and absorbance at 280 nm (----) were measured.

the procedure employed previously[14] in order to isolate the ascites factor (AF) stimulating TK activity in the liver from the ascitic fluid of the Ehrlich ascites tumor-bearing mouse. The frozen effusion collected previously was thawed and centrifuged again at 15,000g for 15 min to remove the remaining cell debris. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and applied to a DEAE-Sephacel column (1×14 cm) equilibrated with the same buffer. Chromatography was performed with a linear gradient of NaCl concentration from 0 to 0.3 M in the same buffer. As shown in Fig. 2, the stimulatory activity for [^{3}H]thymidine uptake into the isolated hepatocytes was detected in the fractions eluted at about 0.1 M NaCl concentration. The active fractions were pooled and concentrated into the AS:40-70 fraction as described above. The final precipitate was dissolved in the same phosphate buffer as described above and applied to a Sephadex G-150 column (1.6×87 cm). As shown in Fig. 3, the stimulatory activity was detected in the fraction corresponding to a relative molecular weight (Mr) of about 60,000, estimated by the use of marker proteins. These active fractions were pooled, dialyzed against 10 mM phosphate buffer (pH 7.0) containing 20% ammonium sulfate and applied to a Phenyl Sepharose CL-4B column (1.2×6.0 cm) equilibrated with the same buffer together with ammonium sulfate.

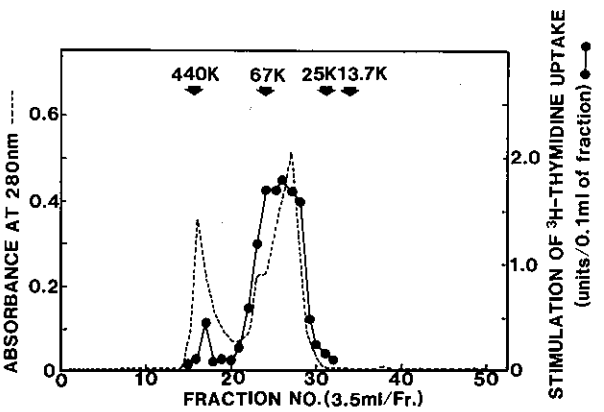

Fig. 3. Sephadex G-150 column chromatography. The active fractions eluted from the DEAE-Sephacel column (Fig. 2) were concentrated as described in the text and fractionated by Sephadex G-150 column chromatography using 10 mM phosphate buffer (pH 7.0). Every 3.5 ml of the eluate was collected in a fraction, and stimulatory activity for [^{3}H]thymidine uptake into free hepatocytes of 0.1 ml of each fraction (●) and absorbance at 280 nm (----) were measured. Ferritin (440,000), BSA (67,000), chymotrypsinogen A (25,000) and ribonuclease A (13,800) were used as marker proteins for molecular weight.

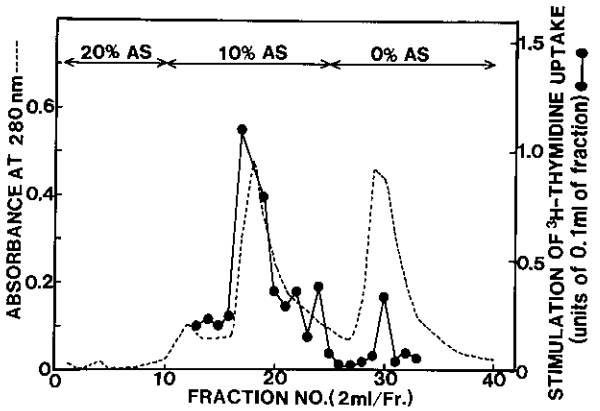

Fig. 4. Phenyl Sepharose CL-4B column chromatography. The active fractions eluted from Sephadex G-150 gel filtration (Fig. 3.) were dialyzed against 10 mM phosphate buffer (pH 7.0) containing 20% ammonium sulfate and applied to the Phenyl Sepharose CL-4B column. The chromatography was performed as described in the text and every 2 ml of the eluate was collected in a fraction. The stimulatory activity for [^{3}H]-thymidine uptake into free hepatocytes of 0.1 ml of each fraction (●) and absorbance at 280 nm (----) were measured. "AS" represents ammonium sulfate.

Table I. Purification of the Factor Stimulating [^{3}H]Thymidine Uptake into Free Hepatocytes from the Ascitic Fluid of an Endometrial Cancer Patient

	Total activity (units)	Yield (%)	Total protein (mg)	Specific activity (units/mg protein)	Purification (-fold)
Crude ascites[a]	3232.6	100.0	2364.1	1.4	1.0
DEAE-Sephacel	2189.4	67.7	346.5	6.3	4.6
Sephadex G-150	359.1	11.1	25.1	14.3	10.4
Phenyl Sepharose CL-4B	187.8	5.8	2.1	89.4	65.3

a) Purification was started from about 50 ml of ascitic fluid.

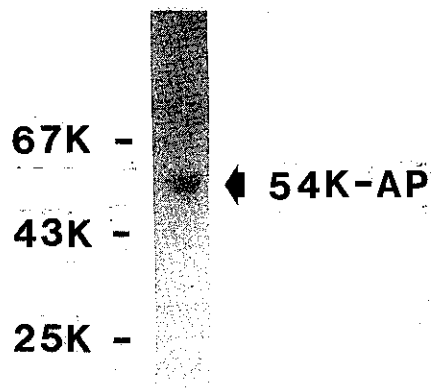

Fig. 5. SDS-PAGE of the purified factor (54K-AP). Electrophoresis of the purified factor eluted from the Phenyl Sepharose CL-4B column was performed as described in "Materials and Methods." BSA (67,000), ovalbumin (43,000) and chymotrypsinogen A (25,000) were used as marker proteins for molecular weight.

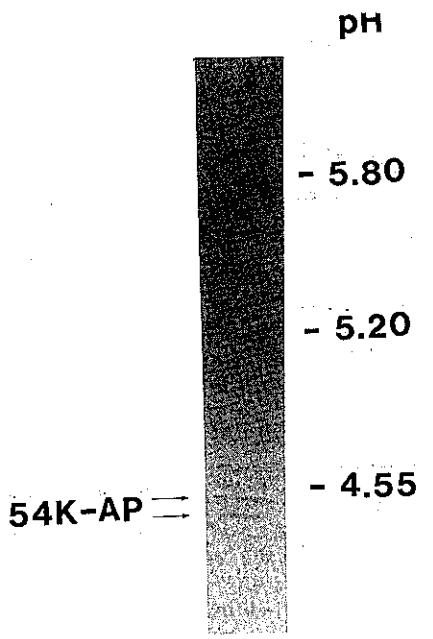

Fig. 6. IEF of 54K-AP. IEF of 54K-AP was performed as described in "Materials and Methods." Soybean trypsin inhibitor (pH 4.55), β-lactoglobulin A (pH 5.20) and bovine carbonic anhydrase (pH 5.85) were used as pI marker proteins.

The column was successively washed with the same buffer, 10 m*M* phosphate buffer containing 10% ammonium sulfate, and then plain 10 m*M* phosphate buffer. The stimulatory activity was detected in the fraction eluted by the elution buffer containing 10% saturated ammonium sulfate (Fig. 4).

The results of purification of the factor stimulating [^{3}H]thymidine uptake into free hepatocytes are summarized in Table I. The specific activity of the finally purified factor had been increased 65.3-fold with 5.8% recovery of the total activity of the ascitic fluid.

The purified factor was dialyzed against 10 m*M* phosphate buffer (pH 7.0) and showed a single protein band stained by Coomassie Brilliant Blue with *Mr* 54,000 on SDS-PAGE under reducing conditions (Fig. 5). Thus, we tentatively designated the protein factor as 54K-ascitic protein (54K-AP). As shown in Fig. 6, 54K-AP exhibited two distinct bands at pH 4.48 and 4.42 in IEF. The amino acid sequence of 54K-AP near the amino-terminal was very similar to that of human α_1-AT[15, 16] (Fig. 7). The two proteins differed from each other in only two amino acids at the 2nd (Asp in α_1-AT) and 15th (His) positions of the 15 amino acids investigated from the amino-terminal, at which 54K-AP contained to His and Leu, respectively. By immunodiffusion analysis, a precipitin line was detected between 54K-AP and both non- and double-diluted preparations of rabbit anti-human α_1-AT antibody (Fig. 8).

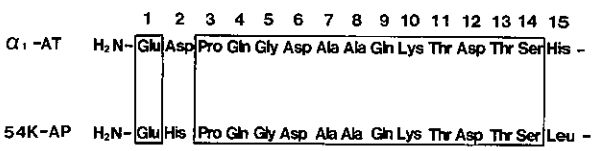

Fig. 7. Comparison of the 15 amino acids at the amino terminal of 54K-AP and human α_1-AT. Amino acid sequencing was performed as described in "Materials and Methods." The sequence of human α_1-AT is from Carrell *et al.*[15] Identical amino acids are those within the two boxes.

Comparison of the properties of 54K-AP and human α_1-AT As shown in Fig. 9, 54K-AP and human α_1-AT, which had been prepared by purification of commercial α_1-AT through DEAE-Sephacel and Affi-Gel blue column chromatography, had 9.5- and 2.5-fold greater stimulatory activity than BSA, respectively. 54K-AP as well as human α_1-AT inhibited casein digestion by either bovine trypsin or α-chymotrypsin (Fig. 10 A, B). 54K-AP also inhibited the PA activity in the conditioned medium of Ishikawa cells in a dose-dependent manner, while human α_1-AT did not cause such inhibition up to the concentration of 0.4 mg/ml in the assay, though with 1.6 mg/ml, 50% inhibition was seen (Fig. 11).

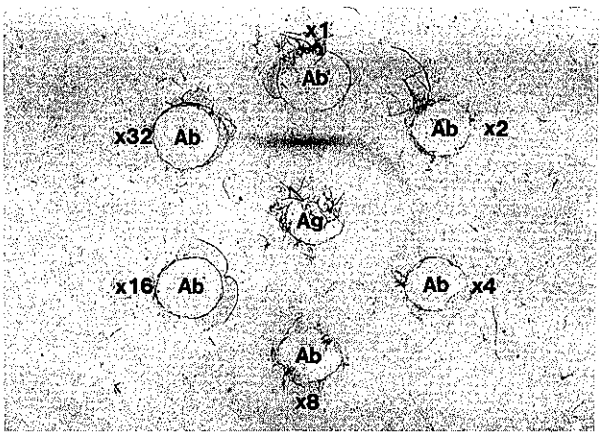

Fig. 8. Double-immunodiffusion analysis of 54K-AP. 54K-AP (0.3 μg) was applied to the spot ("Ag") in the center of the agarose plate. The diluted ($\times 1 \sim \times 32$) anti-human α_1-AT antibody was applied to the "Ab" spots surrounding the "Ag" spot. After incubation at 4°C overnight, the plate was washed with saline and the resulting precipitin was stained with Coomassie Brilliant Blue R-250.

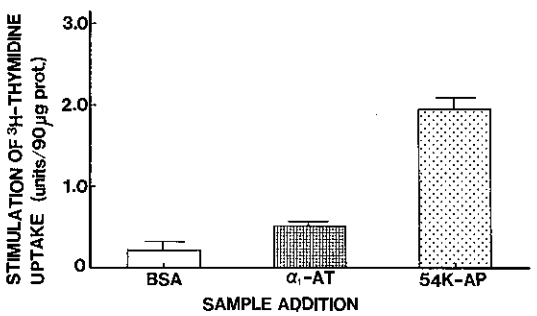

Fig. 9. Comparison of the stimulations of [^{3}H]thymidine uptake into free hepatocytes by 54K-AP and α_1-AT. Stimulation of [^{3}H]thymidine uptake into free hepatocytes by 90 μg of 54K-AP or α_1-AT was measured in triplicate, and compared with the activity of the same amount of BSA as a control. Columns and bars are means and standard deviation.

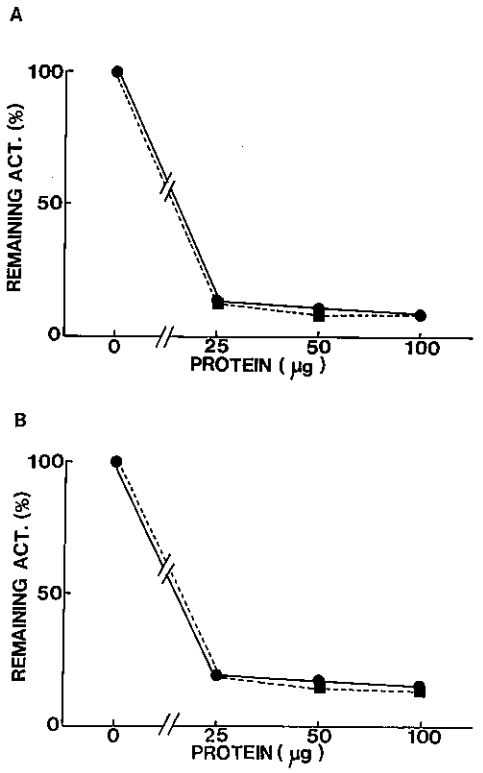

Fig. 10. Comparison of 54K-AP with α_1-AT as regards trypsin-inhibitory activity (A) and α-chymotrypsin-inhibitory activity (B). Various amounts of 54K-AP (●) and α_1-AT (■) were incubated with either 150 μg of bovine pancreatic trypsin (A) or 25 μg of bovine pancreatic α-chymotrypsin (B), and then the remaining activities of the enzymes were measured as described in "Materials and Methods."

DISCUSSION

In this study, 54K-AP, which is quite similar to human α_1-AT in terms of its characteristics (Figs. 5, 6, 7 and 8) was isolated from the ascitic fluid of an endometrial cancer patient (Table I) in essentially the same manner as from Ehrlich ascites tumor-bearing mouse, by monitoring the stimulation of [^{3}H]thymidine uptake into free hepatocytes prepared from a normal mouse liver.

[^{3}H]Thymidine uptake was measured in terms of the radioactivity incorporated into the acid-insoluble fraction, not that in the whole homogenate of the hepatocytes, for convenience. In fact, changes in radioactivity in the whole homogenate paralleled those in the acid-insoluble fraction in the liver of the mouse after transplantation of Ehrlich ascites tumor, as well as after treatment with AF.[4] However, it is still not clear just how the uptake of thymidine is stimulated.

While AF induced about a 3-fold increase in TK activity in the mouse liver *in vivo,* partially purified 54K-AP did not have this effect in the mouse liver (data not shown). Taking into consideration the fact that the increase of TK activity was found in the liver of mice bearing not only a rapidly growing ascites tumor such as the Ehrlich ascites tumor, but also B-16 melanoma, which is a slowly growing solid tumor like most human cancers,[17] we speculate that the TK activity might increase in the liver of endometrial cancer patients, and that 54K-AP might contribute to the stimulation of TK activity.

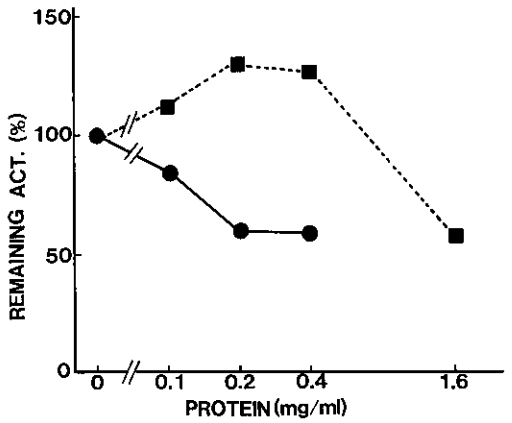

Fig. 11. Comparison of the PA-inhibitory activities by 54K-AP and α_1-AT. Various amounts of 54K-AP (●) and α_1-AT (■) were incubated in the PA assay mixture, and then the remaining PA activity was measured as described in "Materials and Methods."

A structural comparison of 54K-AP and human α_1-AT revealed only slight differences in the amino acid sequence in the amino-terminal region as far as investigated (Fig. 7). There are several well-established polymorphisms[18-20] of human α_1-AT; that is, several mutations of human α_1-AT have been reported. However, fewer mutations have occurred in the amino-terminal region than in the carboxyl-terminal region.[19] The differences in the amino acid sequence in the amino-terminal region of 54K-AP are considered to be reliable in the light of current amino acid sequencing technology.

We found that 54K-AP showed a single band on SDS-PAGE (Fig. 5), like human α_1-AT, and two bands on IEF (pH 4.48 and 4.42) (Fig. 6), slightly more acidic than those of human α_1-AT (pH 4.55 and 4.50).[21] It has been reported that the microheterogeneity of human α_1-AT is due to non-genetic variation in the branched oligosaccharide side chain.[22,23] In addition, Thompson *et al.*[24] have found fucosylated forms of α_1-AT in the sera of ovarian cancer patients. The nature of the microheterogeneity of 54K-AP still remains to be clarified.

54K-AP inhibited various protease activities (Figs. 10 and 11). Chawla *et al.*[25,26] reported that the titer of anti-α_1-AT antibody increased in the sera of cancer patients, but the trypsin-inhibitory activities did not increase proportionally, and so they assumed that a portion of the circulating α_1-AT in the patients was functionally inert. Since 54K-AP could inhibit trypsin activity as well as human α_1-AT (Fig. 10 A), it is obviously not the same inert form of α_1-AT that Chawla *et al.* found.

Several investigators have reported a stimulated fibrinolytic activity in ascites[27,28] and an increased amount of α_1-AT in the sera of human ovarian cancer patients.[24] Clemmensen and Christensen[29] reported that purified human α_1-AT formed a complex with urokinase to inhibit the activity. As regards inhibition of PA, which was released into the conditioned medium from human endometrial cancer Ishikawa cells, 54K-AP seemed to function somewhat more effectively than α_1-AT (Fig. 11).

It has been reported that several factors derived from tumor cells stimulate the activities of TK,[30] ornithine decarboxylase[31] and alkaline phosphatase.[32] On the other hand, the stimulatory activity of [^{3}H]thymidine uptake into hepatocytes was not detected in the conditioned medium of endometrial cancer Ishikawa cells (data not shown), suggesting that 54K-AP was not a product of the cancer cells, but rather of the normal tissues of cancer patients. Moreover, a much more stimulated [^{3}H]thymidine uptake activity was detected in the serum of an endometrial cancer patient than in normal serum (data not shown). Since plasminogen activator is known to be concerned with tumor invasion and metastasis,[33] 54K-AP may play some defensive role in cancer patients. However, 54K-AP had neither a stimulatory nor an inhibitory effect on the growth of Ishikawa cells (data not shown), and its possible effect on the growth of other cancer cells and normal cells is now under investigation. The present results at least raise the possibility that 54K-AP may be useful as a specific marker of gynecological cancers.

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