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New human colorectal carcinoma cell lines that secrete proteinase inhibitors in vitro

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Summary. Two new human cell lines, RCM-1 and CoCM-1, have been established from primary colorectal adenocarcinomas. Both cell lines were unique in that the cultures secreted trypsin inhibitors in vitro. The activities of these inhibitors were accumulated in serum-free media of both cell lines over a period of several days. Two inhibitors (PI-1 and PI-2) were isolated from serum-free conditioned medium in which RCM-1 was grown by anion-exchange and gel filtration high-performance liquid chromatography. PI-1 inhibited trypsin and chymotrypsin strongly, and pancreatic elastase weakly. Its molecular weight was about 57 kilodaltons (Kd) as determined by gel filtration chromatography. It cross-reacted with the antiserum elicited against human α_1-antitrypsin in double immunodiffusion. PI-1 corresponding to α_1-antitrypsin was also demonstrated immunohistochemically in both cell lines. PI-2 inhibited trypsin strongly, and chymotrypsin, kallikrein and plasmin weakly. It had higher molecular weight (200–300 Kd) than that of PI-1, and did not cross-react with antisera against human α_1-antitrypsin, α_2-macroglobulin, α_1-antichymotrypsin, α_2-plasmin inhibitor, inter-α-trypsin inhibitor and urinary trypsin inhibitor. RCM-1 and CoCM-1 are the first colorectal adenocarcinoma cell lines that secrete functionally active trypsin inhibitors, including α_1-antitrypsin in vitro, and are useful for the study of tumor-cell derived proteinase inhibitors.

Key words: Colorectal carcinoma cell lines – Proteinase inhibitors – Trypsin inhibitors – α_1-antitrypsin

Introduction

Increased trypsin inhibitors in the serum or tumor tissues from various cancer patients have been described (Harris et al. 1974; Cooper et al. 1976; Matsuda et al. 1983; Okumichi et al. 1984; Cheung and Lau 1986; Chawla et al. 1987; Sawaya et al. 1987). In particular, α_1-antitrypsin (α_1-AT), a serine proteinase inhibitor which accounts for 90% of the trypsin inhibitory capacity of normal human plasma, has been recently demonstrated immunohistochemically in various tumor cells (Palmer et al. 1976; Kittas et al. 1982; Aroni et al. 1984; Wittekind et al. 1986; Zuccarello et al. 1987). However, its source, role and significance in tumors are not known with certainty. It is difficult to distinguish between production of α_1-AT by tumor cells and absorption of the material by tumor cells. It also remains to be determined whether or not α_1-AT stained in tumor cells is functionally active.

While detailed biochemical study of tumor cell-derived biomolecules is difficult to perform in vivo, serum-free culture of tumor cells provides an excellent source in vitro. To determine the trypsin inhibitors produced by the tumor cells themselves, we have tried to establish a new cell line of human colorectal carcinoma, one of the commonest solid tumors. In the present work we wish to describe the establishment of two new human colorectal carcinoma cell lines that secrete functionally active trypsin inhibitors in vitro. Thus far, there are a large number of cell lines derived from colorectal carcinomas, but none of these have secreted trypsin inhibitors in vitro. We also reported the partial purification and some characterization of trypsin inhibitors in the conditioned medium.

Materials and methods

Establishment of cell lines. RCM-1 was derived from a primary carcinoma of the rectum with histologic diagnosis of well differentiated adenocarcinoma, removed from a 73-year-old female in May, 1984. CoCM-1 was derived from a moderately differentiated adenocarcinoma of the descending colon removed from a 45-year-old male in November, 1984. Fragments of the tumors were rinsed repeatedly with phosphate buffered saline (PBS) and minced finely. They were incubated at 37° C in

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Hanks' balanced salt solution supplemented with dispase (1500 U/ml; Gohdoh Shusei. Co., Tokyo, Japan), 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), kanamycin (250 μg/ml), streptomycin (200 μg/ml), penicillin G (100 U/ml) and 25 mM N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid (HEPES), pH 7.25, until tissue fragments were dispersed. The cells were recovered by centrifugation at 55 g for 5 min and seeded in 25-cm^2 flasks in growth medium, a mixture of RPMI 1640 and Ham's F-12 (1:1; Nissui Seiyaku Co, Tokyo, Japan) supplemented with 10% FBS, streptomycin (90 μg/ml) and penicillin G (90 U/ml). Then, the flasks were cultured in a fully humidified atmosphere of 5% CO_2 in air at 37° C. Fibroblasts were removed mechanically by scraping cultures and by differential trypsinization as described by Brattain et al. (1981). The cultures were passaged with 0.125% trypsin and 0.5 mM EDTA at a split ratio of 1:2. The cell cultures were free from mycoplasma contamination when tested in mycoplasma medium (MYCOTRIM, Hana Media Inc, Berkeley, USA) and DNA-binding fluorochrome stain using 4′,6-diamino-2-phenylindole (Boehringer Mannheim, FRG).

Growth characteristics. Replicate 35-mm dishes were seeded at 2×10^5 cells/3 ml growth medium. The number of viable cells was counted daily. Doubling time was determined during the log phase of growth. Throughout the entire procedure, cell viability was determined by means of the trypan blue exclusion method.

Plating efficiency was determined by seeding 5×10^2 cells in 60-mm dishes using 5 ml of the growth medium. The number of visible colonies was counted 10 days later, after methanol fixation and staining with Giemsa.

Cell culture characterization. For chromosome analysis, cultures were refed with fresh growth medium 24 h prior to the addition of Colcemid (0.1 μg/ml). The cells were disaggregated 3 h later, incubated in 0.2% KCl for 20 min at 37° C and fixed with methanol/acetic acid (3:1). The chromosome specimens were prepared by the ordinary air-drying method and stained with Giemsa.

For electron microscopy, cultured cells and minced xenografts were fixed in 4% formaldehyde and 1% glutaraldehyde on 0.1 M phosphate buffer for 2 h, postfixed in 2% OsO_4 for 1 h. Ultrathin sections were stained with uranyl acetate and lead citrate.

Mucin production was detected by alcian blue staining. Periodic-acid Schiff (PAS) staining was also performed for detection of mucin, glycoprotein and glycogen. For immunohistochemical study, cells were cultured on bovine lens capsules, fixed with buffered formalin and paraffin embedded sections were stained using Vectastain ABC Kit (Vector Laboratories, Burlingame, USA).

Carcinoembryonic antigen (CEA) assays were performed by a sandwich enzyme immunoassay (EIA) kit (Fujirebio Inc, Tokyo, Japan). Amounts of urokinase-type plasminogen (u-PA) and tissue-type plasminogen activator (t-PA) antigens were measured by sandwich EIA (Suzumiya et al. 1988).

Xenografts in nude mice. Tumorigenicity was tested by inoculating 5×10^6 cells s.c. into the back of male athymic mice with a BALB/c genetic background (Clea Japan, Tokyo, Japan). Passage of the xenografts were performed by inoculating the minced tumor tissues into the back of the mice. Tumor volume was calculated weekly by using the formula, length × $(\text{width})^2$ × 0.5. Formalin-fixed paraffin-embedded sections of xenografts were stained with hematoxylin-eosin or used for the immunohistochemical study.

Preparation of serum-free conditioned medium. To maintain the cells under serum-free condition, confluent cultures were washed three times with PBS and replaced with a serum-free medium [a mixture of RPMI 1640 and Ham's F-12 (1:1) supplemented with L-glutamine (746 μg/ml), Na_2SeO_3 (2.6 ng/ml), streptomycin (90 μg/ml), penicillin G (90 U/ml), and 25 mM HEPES, pH 7.3] or the serum-free medium supplemented with insulin (10 μg/ml, Collaborative Research Inc, Waltham, USA) and transferrin (10 μg/ml, Collaborative Res.) (SFIT medium).

To collect serum-free conditioned medium (SFCM), RCM-1 and CoCM-1 cells were cultured in 75-cm^2 flasks in 20 ml of the growth medium. Seven days after the cells reached confluency, the flasks were rinsed three times with PBS and replaced with 18 ml of the serum-free medium. SFCMs were harvested and cultured RCM-1 and CoCM-1 cells were refed by fresh serum-free medium at 2-day intervals for 10 days and 4 days, respectively. The media collected during the first 2 days were discarded while subsequent harvests were collected, centrifuged (2,000 *g*, 20 min) and stored at −40° C. A large pool of SFCM was thawed, and concentrated by ultrafiltration through YM-5 Diaflo membrane (Amicon Far East Ltd., Tokyo, Japan). The concentrated SFCM was centrifuged (4,500 *g*, 20 min), lyophilized, and stored at −20° C until analyzed.

Column chromatography. High-performance liquid chromatography (HPLC) was performed with a Pharmacia's FPLC system (Pharmacia Fine Chemicals, Uppsala, Sweden). Anion-exchange HPLC fractionation was performed on a Mono Q HR5/5 column (0.5 × 5 cm, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.7. Gel filtration HPLC fractionation was performed on a Superose 12 column (1 × 30 cm, Pharmacia) equilibrated with 50 mM Tris-HCl, containing 200 mM NaCl, pH 7.6. Concanavalin A (Con A) affinity HPLC fractionation was performed on a LA-Con A column (0.46 × 15 cm, Seikagakukogyo Co, Tokyo, Japan) equilibrated with 50 mM Tris-HCl, pH 7.2, containing 200 mM NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. Bound protein was eluted with a linear gradient of 0 to 100 mM α-methyl-D-glucoside in the initial buffer.

Proteinase, inhibitor and protein assay. Trypsin (type I, Sigma Chemical Co, St. Louis, USA), α-chymotrypsin (type I-S, Sigma) and papain (type III, Sigma) were measured using ^{3}H-hemoglobin substrate at 37° C for 3 h described in a previous work (Ishihara et al. 1986). Trypsin and chymotrypsin were assayed in 100 mM Tris-HCl/15 mM $CaCl_2$, pH 7.7, and papain in 100 mM phosphate/0.1 mM cysteine and EDTA, pH 6.3. Trypsin was also measured against Bz-L-Arg-pNA (L-BAPNA) (Sigma) (Somorin et al. 1979). Porcine elastase (Sigma), human plasmin (Green Cross Co, Osaka, Japan), u-PA (Green Cross), porcine pancreatic kallikrein (Sigma) and thrombin (Daiichi Pure Chemical Co, Tokyo, Japan) activities were measured against Suc-$(\text{L-Ala})_3$-pNA (Sigma), H-D-Val-Leu-Cys-pNA (S-2251), pyro-Glu-Gly-Arg-pNA (S-2444), H-D-Val-Leu-Arg-pNA (S-2266) and H-D-Phe-Pipq-Arg-pNA (S-2238) Kabi Diagnostica, Stockholm, Sweden), respectively (Bieth et al. 1974; van Dam-Mieras et al. 1984; Friberger 1982).

The inhibitory activities of the sample on various enzymes were determined from the residual activities of the enzymes after preincubation with a mixture of enzyme and various amount of inhibitors at 37° C for 15 min and were expressed as percentage inhibition. The trypsin inhibitory activity was estimated by the amount of protein which inhibited 50% activity of 0.1 μg of trypsin (ID_{50}) and arbitrarily refers to 0.1 inhibitory unit (Inh. U).

The protein concentration of samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Antiserum preparation. To obtain anti-urinary trypsin inhibitor rabbit serum, rabbits were injected in the foot pads with 1 mg of human urinary trypsin inhibitor (UTI) (Green cross) emulsified with complete Freund's adjuvant followed by booster injections of the same amount of antigen in the dorsal muscles three times at 14-day intervals. Ten days after the last booster inoculation the animals were exsanguinated and the sera collected. The IgG fraction was separated from serum by Na_2SO_4 fractionation (18% saturation) and DEAE-cellulose column chromatography. Rabbit IgG against human α_1-AT, α_1-antichymotrypsin, α_2-macroglobulin, inter-α-trypsin inhibitor, α_2-plasmin inhibitor, and bovine whole serum protein were obtained from DAKO (DAKOPATTS, Glostrup, Denmark).

Results

Characteristics of two cell lines

RCM-1 cell line. Primary cultures were fed regularly by partially changing the medium. Fibroblasts proliferated rapidly and had to be repeatedly removed until they became senescent. Initial cell passage was delayed until heavy tumor cell growth was observed. Time to first passage was 5 months. Cells had reached passage 8 after 1 year in vitro. After this time, subculturing was carried out every 2 or 3 weeks. Cultured RCM-1 cells exhibited typical epithelial features (Fig. 1 A). Some of them contained cytoplasmic vacuoles which were positive with the alcian-blue stain. Confluent monolayers formed many domes resembling those observed in cultures of well differentiated colonic carcinoma cells (Park et al. 1987). They survived and retained the ability to form domes in the serum-free medium for at least 20 days (Fig. 1 A). Material contained in the domes was strongly PAS-positive, but was almost negative with alcian-blue. The doubling time was calculated at 71.4 h, and the plating efficiency was 53.2% using cells from passage 9. Modal chromosome number at 9th passage was between 71 and 73 with considerable scattering in counts. SFCM from the confluent cultures contained 58.5 ng of CEA/10^6 cells/24 h. The amount of CEA in SFCM was unchanged at least for 10 days (Fig. 2). Therefore, confluent RCM-1 cells maintained under the serum-free condition not only survived for long periods but also retained their capacity to secrete tumor-cell derived products. RCM-1 cells (30th passage) secreted about 7.7 ng of u-PA antigen/10^6 cells/24 h at the log phase of growth. T-PA antigen was not detectable in the conditioned medium (detection limit; 0.01 ng/150 µl). Tumor grown in the nude mouse was lobular, well demarcated and histological examination showed it to be well differentiated adenocarcinoma resembling the original tumor (Fig. 1 B). The most characteristic ultrastructural features were uniform microvilli with prominent filamentous core and well formed junctional complexes either in vivo or in vitro (Fig. 1 C, D). Volume doubling time of xenografts was 204 ± 36 h. Metastases were not observed.

CoCM-1 cell line. Initial growth of the primary culture of CoCM-1 was slow, but contamination with fibroblasts was minimal. Tumor cells grew in patches and the culture did not attain a complete monolayer for 3 months; at this time subculturing was performed. Cultured CoCM-1 cells exhibited epithelial features with a tendency to pile up (Fig. 1 E). Unlike RCM-1, only a few cells contained alcian-blue-positive mucin in their cytoplasm and dome formation was not observed. The doubling time was 45 h. The plating efficiency was 6% using cells from passage 5 and 25% using cells passage 28. Modal chromosome number at 28th passage ranged from 65 to 70. Confluent CoCM-1 cells survived in SFIT medium at least for 10 days, and secreted 45.5 ng of CEA/10^6 cells/24 h. Tumor grown in the nude mouse was moderately differentiated adenocarcinoma resembling the original tumor (Fig. 1 F). Volume doubling time was 185 ± 19 h. Metastases were not observed.

Trypsin inhibitory activity in SFCM

SFCM of RCM-1 (passage No. 38–42) contained 11.6 µg of protein/ml of medium and its ID_{50} was 2.1 µg (47.6 Inh. U/mg). SFCM of CoCM-1 (32nd passage) contained 10.2 µg of protein/ml and its ID_{50} was 13.8 µg (7.3 Inh. U/mg). As shown in Fig. 3, RCM-1 cells at passage 40 secreted about 0.72 and 0.8 Inh. U of trypsin inhibitor/10^6 cells/24 h into the serum-free medium and SFIT medium, respectively. CoCM-1 cells (passage 34) secreted 0.34 Inh. U/10^6 cells/24 h into SFIT medium at least for 10 days.

Partial purification of trypsin inhibitors

The lyophilized SFCM of RCM-1 cells from passages 38–42, was dissolved in distilled water and filtrated through a prepacked column PD-10 (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.7. Five ml (2.5 mg protein) was applied to a Mono Q HR 5/5 column and eluted with a linear gradient of NaCl. Trypsin inhibitory activities were found in two fractions and designated as PI-1 and PI-2 (Fig. 4 A). A minor activity was present in the breakthrough fraction. When SFCM of CoCM-1 was applied on the Mono Q column

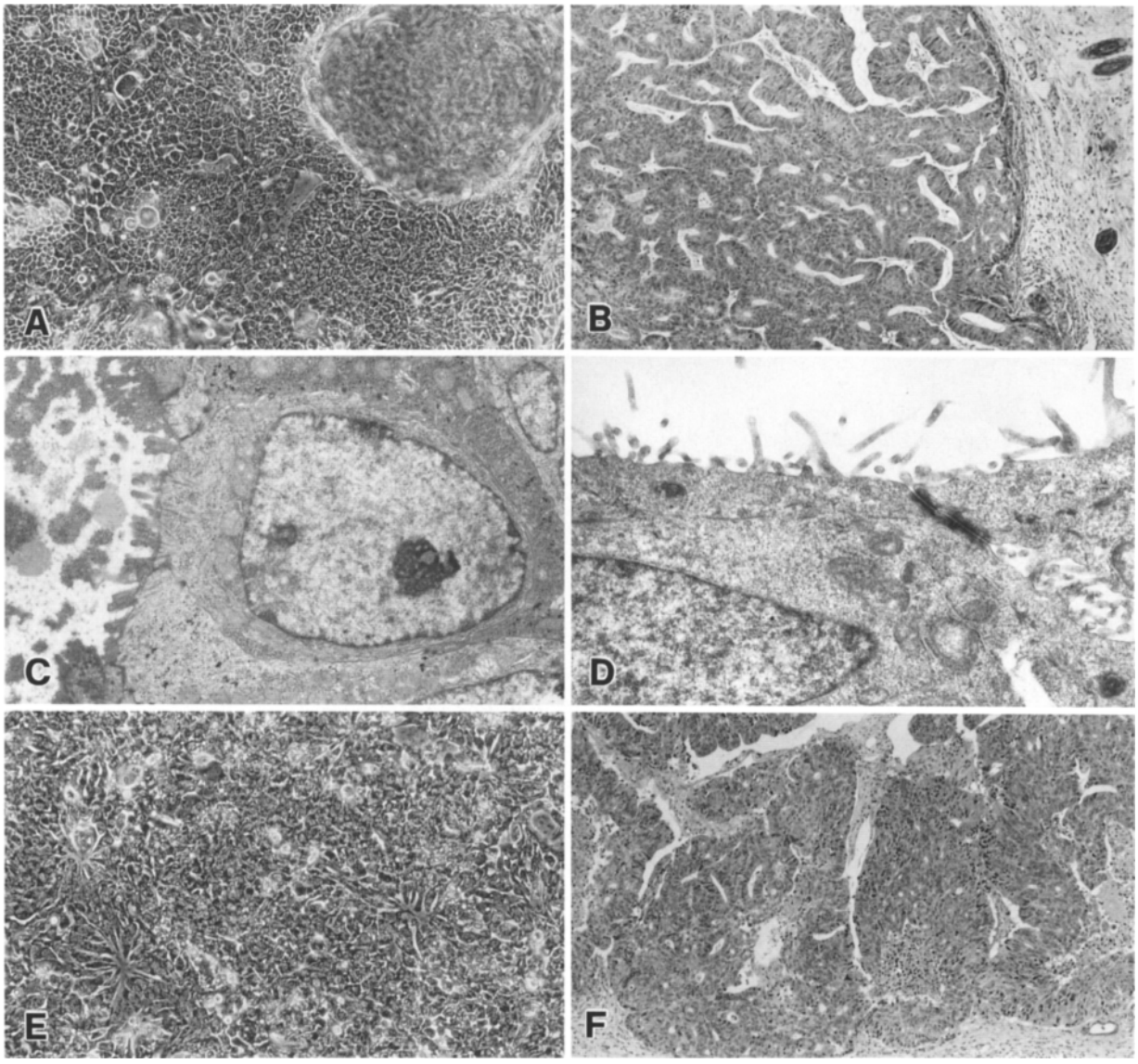

Fig. 1 A–F. Morphology of RCM-1 and CoCM-1 cells. **A** Phase-contrast micrograph of confluent monolayer of cultured RCM-1 cells (passage 38) maintained in the serum-free medium for 12 days. Raised domes are out of focus while cells tightly adhering to the substrate are in focus. **B** Xenograft of RCM-1 (×58, hematoxylin-eosin). **C** Transmission electron micrographs of RCM-1 cells grown in nude mice (×4.500) and **D** on plastic culture flasks (×9.800). **E** Phase-contrast micrograph of CoCM-1 cells in the growth medium (×92). **F** Xenografts of CoCM-1 (×58, hematoxylin-eosin)

under the same condition, the trypsin inhibitory activities were eluted into almost similar position, but the activities were less potent than those of RCM-1 (Fig. 4B).

PI-1 and -2 were pooled and concentrated with YM-5 Diaflow membrane. Then 200 µl of each sample was applied to a Superose 12 column and eluted (Fig. 5). Inhibitory activity of PI-1 was eluted in a single peak between bovine serum albumin and ovalbumin and that of PI-2 was noted in almost similar region of catalase, which indicated its high molecular weight.

Some properties of trypsin inhibitors

The molecular weight of PI-1 was estimated to be 57 Kd by gel filtration chromatography. It inhibited trypsin, chymotrypsin, elastase, kallikrein and thrombin, but not u-PA or papain. Its ID_{50} was 0.57 µg (Table 1, Fig. 6). The values of commercially obtained human α_1-AT (Sigma) and UTI were 0.32 and 0.075 µg, respectively, in the same assay. PI-1 crossreacted with antiserum elicited against human α_1-AT (Fig. 7). The reactivity of the antiserum was not diminished even by satura-

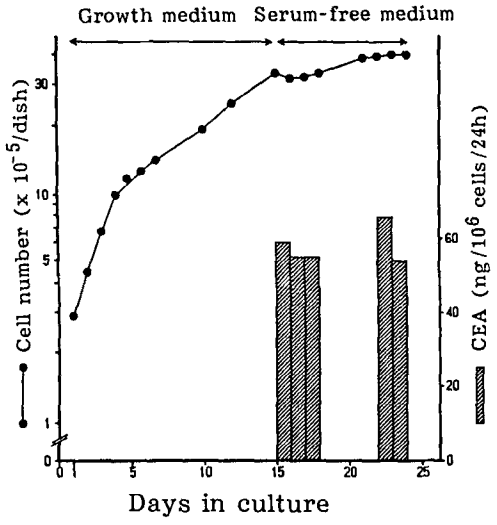

Fig. 2. Growth curve of RCM-1 cells. Confluent cells were maintained in the serum-free medium and released CEA was assayed

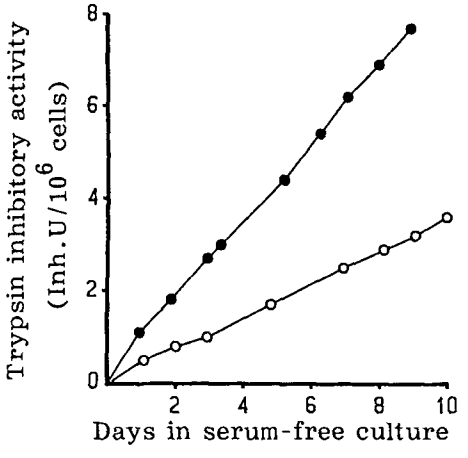

Fig. 3. Release and accumulation of trypsin inhibitors during serum-free maintenance of RCM-1 (●—●) and CoCM-1 (○—○) cells. Confluent cultures (25 cm^2 flasks) were washed and replenished with 5 ml SFIT medium. Conditioned media were harvested and the cultures were refed at 1-to 2-day intervals. Each point represents the mean value from 2 flasks

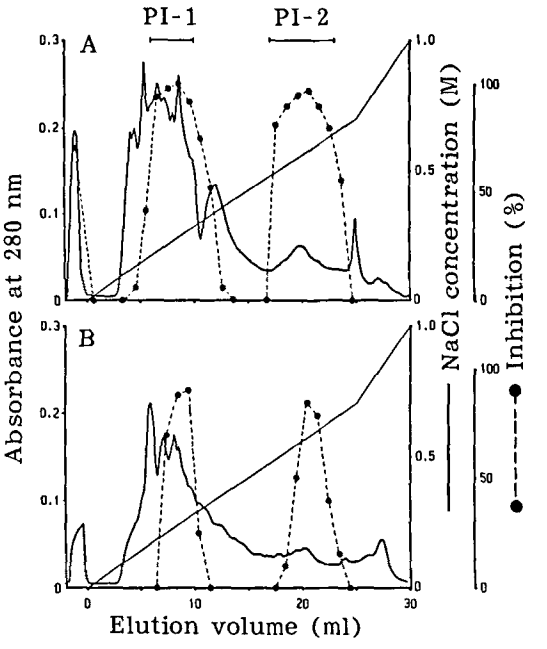

Fig. 4. Chromatography of SFCM on a Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl, pH 7.7, and eluted with a linear gradient of NaCl at flow rate of 1 ml/min. Fractions of 1 ml each were collected and inhibitory activity of each fraction (**A**:50 µl, **B**: 100 µl) against 0.1 µg trypsin was assayed. **A** SFCM of RCM-1, 2.5 mg of protein dissolved in 5 ml of the initial buffer was applied. Fractions containing the activity were pooled (|—|), concentrated and designated as PI-1 and PI-2. **B** SFCM of CoCM-1, 1.14 mg of protein was applied

Table 1. Inhibitory effects of RCM-1 derived proteinase inhibitors

Enzyme	Substrate	Protein concentration causing 50% inhibition (µg)	
		PI-1	PI-2
Trypsin (1.0 µg)	Hemoglobin	0.56	0.08
	L-BAPNA	0.57	0.09
Chymotrypsin (0.1 µg)	Hemoglobin	0.86	0.8
Pancreas elastase (0.1 µg)	Suc-$(Ala)_3$-pNA	1.22	NI[a]
Pancreas kallikrein (0.1 µg)	S-2266	3.0	0.75
Thrombin (0.41 nKat)	S-2238	9.3	NI
Plasmin (0.005 unit)	S-2251	11.5	1.7
u-PA (0.1 µg)	S-2444	NI	NI
Papain (0.1 µg)	Hemoglobin	NI	NI

[a] NI; no inhibition

tion of the antiserum with FBS. Furthermore, the antiserum did not crossreact with FBS. Thus, it is evident that isolated RCM-1-associated α_1-AT is not derived from bovine serum. Interestingly, when PI-1 was eluted on a Con-A column, about 90% of the recovered activity was washed off with starting buffer. The Con A unbound protein also crossreacted with the antiserum against α_1-AT (Fig. 7) and its ID_{50} was 0.17 µg. Additionally, most protein (86%) of the commercially obtained human α_1-AT had an ability to bind to the Con A column under the same condition. PI-1 was heat-labile and its activity was reduced to 42% at 60° C for 60 min and to 0% at 80° C for 30 min, pH 7.6.

The molecular weight of PI-2 was relatively high (200–300 Kd) by gel filtration chromatogra-

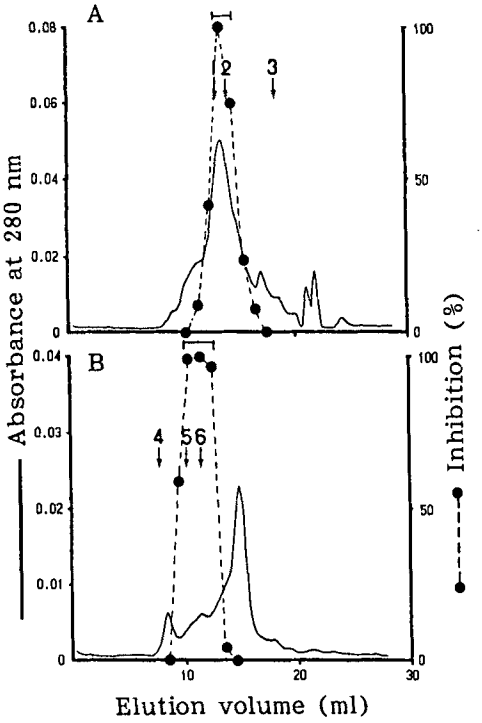

Fig. 5. Gel filtration of the inhibitory fractions on a Superose 12 column. 200 µl of the inhibitory fractions from Mono Q column was eluted with 50 mM Tris-HCl/200 mM NaCl, pH 7.6, at a flow rate of 0.5 ml/min. Fractions were collected every 2 min and inhibitory activity against 0.1 µg trypsin (●---●) of each fraction (**A**: 100 µl, **B**: 300 µl) was assayed. Samples: **A** PI-1, 1200 µg/ml; **B** PI-2, 60 µg/ml. Arrows indicate the position of the standards, 1) bovine serum albumin (67 Kd); 2) ovalbumin (43 Kd); 3) chymotrypsinogen A (25 Kd); 4) thyroglobulin (669 Kd); 5) ferritin (440 Kd); 6) catalase (223 Kd). Active fractions (|——|) were collected and used for the experiments

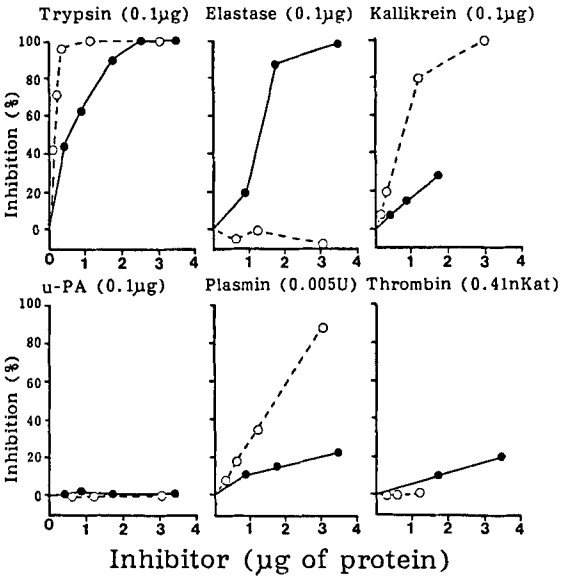

Fig. 6. Effects of PI-1 (●——●) and PI-2 (o---o) on various proteinases. Buffer and substrates used were 100 mM Tris-HCl/15 mM $CaCl_2$, pH 7.7, L-BAPNA for trypsin; 50 mM Tris-HCl, pH 7.4, Suc-(L-Ala)$_3$-pNA for elastase; 50 mM Tris-HCl, pH 8.0, S-2238 for kallikrein; 50 mM Tris-HCl/100 mM NaCl, pH 7.8, S-2266 for thrombin; 15 mM Tris-HCl/12 mM NaCl and 0.01% Tween 80, pH 7.4, S-2251 for plasmin; 50 mM Tris-HCl/38 mM NaCl, pH 8.8, S-2444 for u-PA

phy (Fig. 5). It did not cross-react with antisera elicited against α_2-macroglobulin, inter-α-trypsin inhibitor, α_1-AT, α_1-antichymotrypsin, α_2-plasmin inhibitor, UTI, or bovine whole serum. Furthermore, its activity was not inhibited by the antisera. This inhibitor strongly inhibited trypsin using hemoglobin and L-BAPNA as substrates, but was less potent in inhibiting chymotrypsin, plasmin and kallikrein. It had no effect on u-PA, elastase, thrombin and papain (Table 1, Fig. 6). It was relatively acid and heat resistant. About 96% of the activity remained after incubation at pH 2.0, 37° C, for 60 min, and 90% of the activity remained after incubation at pH 7.6, 80° C for 30 min.

Immunohistochemical study

Presence of α_1-AT in cultured RCM-1 was also demonstrated by immunohistochemical study. Al-

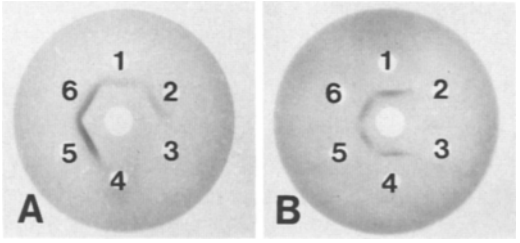

Fig. 7. Double immunodiffusion of anti-human α_1-AT rabbit IgG against PI-1. **A** center well: anti-human α_1-AT rabbit IgG (100 µg/20 µl); peripheral wells:1) PI-1, Superose 12 fraction (2.6 µg, 0.45 Inh. U/10 µl); 2) PI-1, Con A breakthrough fraction (0.5 µg, 0.29 Inh. U/10 µl); 3) PBS (10 µl); 4) FBS (500 µg/10 µl); 5), 6) α_1-AT (1.0, 0.5 µg/10 µl). **B** center well: PI-1, Superose 12 fraction (4.3 µg/20 µl); peripheral wells:1) anti-α_1-AT + FBS (80 µg + 4 mg/10 µl); 2) 3) PBS (10 µl); 4), 5), 6) anti-α_1-AT (30, 50, 80 µg/10 µl)

most all cells had α_1-AT on the surface and some of them in their cytoplasm (Fig. 8 A). Interestingly, intracytoplasmic vacuoles often contained abundant immunoreactive products (Fig. 8 B). Xenograft and original tumor of RCM-1 also contained scattered strongly positive cells and many weakly positive cells. (Fig. 8 C). Some of the cultured CoCM-1 cells showed positive immunohistochemical reaction for α_1-AT, but the reactivity was very weak.

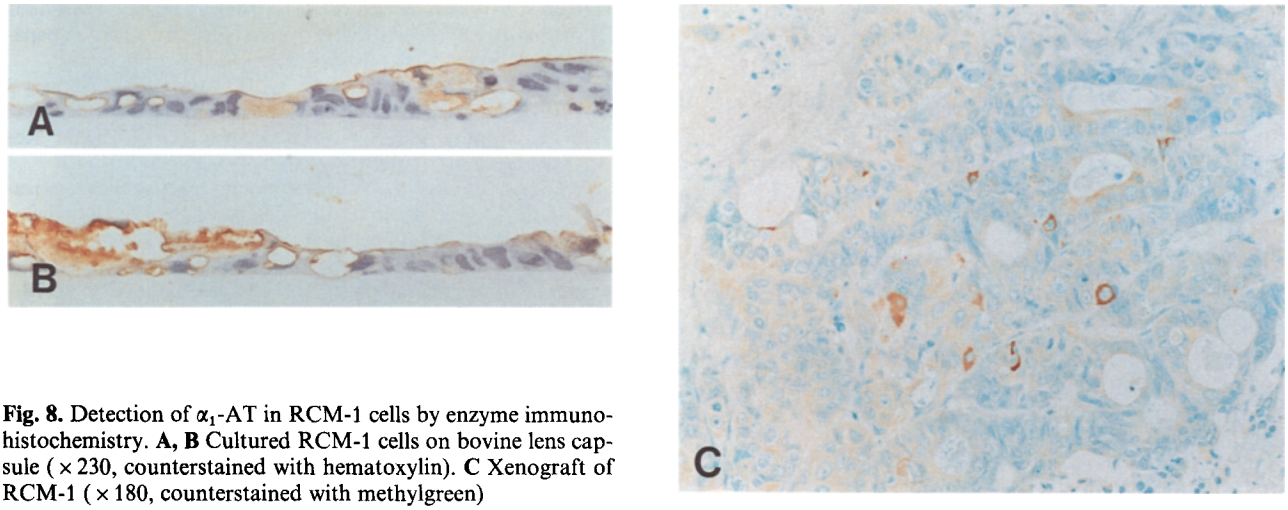

Fig. 8. Detection of α_1-AT in RCM-1 cells by enzyme immunohistochemistry. **A, B** Cultured RCM-1 cells on bovine lens capsule (×230, counterstained with hematoxylin). **C** Xenograft of RCM-1 (×180, counterstained with methylgreen)

Discussion

In the present report we characterized two newly established human colorectal adenocarcinoma cell lines and demonstrated the secretion of proteinase inhibitors in the conditioned media. The original cell lines, designated RCM-1 and CoCM-1, have been propagated continuously by serial passages during the past 43 and 47 months, respectively. RCM-1 in particular still retains some of its original morphological and functional features in vitro, and can be maintained under the serum-free condition for long periods.

Both cell lines secreted at least two trypsin inhibitors (PI-1 and PI-2) separated by anion-exchange HPLC. Both inhibitors inhibited not only trypsin but also other serine proteinase, such as chymotrypsin, plasmin and kallikrein. PI-1 was immunologically similar to human α_1-AT. It also possessed similar antiproteolytic property to α_1-AT; which is classically considered to be produced by hepatocytes. Although, a few human cell lines have been reported to synthesize functionally active α_1-AT in vitro, all of the previous reports concern hepatoma cell lines (Glasgow et al. 1982; Carlson et al. 1984). RCM-1 is the first colorectal carcinoma cell line to secrete active α_1-AT in vitro. Interestingly, whereas most protein of human α_1-AT bound to Con A, the tumor derived α_1-AT failed to bind under the same conditions. Thus, it is suggested that the tumor-derived α_1-AT contained at least two triantennary oligosaccharide sidechains (Vaughan et al. 1982) or abnormaly glycosylated atypical side chains (Carlson et al. 1984). It has been suggested that α_1-AT could be identified immunohistochemically in gastric adenocarcinomas but not in colorectal adenocarcinomas (Kittas et al. 1982). However, Yoshimura et al. (1978) demonstrated human α_1-AT in the serum of nude mice bearing human colon adenocarcinoma by the double immunodiffusion method. Another rectal cancer cell line recently established in our laboratory also secreted trypsin inhibitors in vitro, and the cells were immunohistochemically positive with α_1-AT (unpublished observation). Cheung and Lau (1986) reported the isolation of an inhibitor immunologically related to α_1-AT from tumor tissue extracts of human colorectal carcinomas which had some distinct characteristics from PI-1. The tumor tissue-derived inhibitor did not inhibit elastase but did inhibit u-PA, and had a molecular weight of 66 Kd.

PI-2 was a high-molecular-weight inhibitor whose nature is not clear at present. However, it did not cross-react with antisera against the major trypsin inhibitors and bovine whole serum. The PI-2 fraction contained about 45% of the total inhibitory activity obtained from the Mono Q column, and the accumulation of the total inhibitory activity in SFCM was constant for at least 10 days under serum-free conditions. For the above reasons, the authors believe that this inhibitor is not derived from bovine serum and further characterization of this inhibitor is now underway.

The role and significance of these tumor-derived proteinase inhibitors are not known at present. Considerable evidence has amassed suggesting that proteinase production by tumor cells may be required for invasiveness (Tryggvason et al. 1987). During tumor invasion of the surrounding tissue, the effective and important proteolytic reactions would take place in the narrow pericellular space between invasive tumor cells and matrix, and enzyme confined to such a space may be protected

from circulating proteinase inhibitors. Thus, local concentrations of active proteinase inhibitors may be important in inhibiting tumor proteinases, and in this manner affecting tumor invasiveness (Pauli and Kuettner 1984). In fact, RCM-1 cells also secreted u-PA and high local levels of plasmin induced by the u-PA may be inhibited by the inhibitors described here. Conversely, evidence suggests that tumor cell-derived serine proteinase inhibitors, such as α_1-AT, may modulate the host-tumor immune responses and have immunosuppressive effects. It is known that α_1-AT plays an important role in inhibiting the blastogenic response of normal lymphocytes (Arora et al. 1978) and the cytotoxic reactions of lymphocytes (Ades et al. 1982; Redelman and Hudig 1980), and that lymphocyte granule-mediated cytolysis requires serine proteinase activity (Hudig et al. 1987). Recently, immunohistochemical studies suggested a bad prognosis for patients with α_1-AT-or α_2-macroglobulin-rich tumors (Tahara et al. 1984; Matoska et al. 1988).

Again, little is known of the roles and significance of tumor-derived proteinase inhibitors such as α_1-AT. The cell lines described here provide a readily available and reliable source of material for the study of proteinase inhibitors, including α_1-AT, secreted by human colorectal carcinoma cells.

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