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Characterization of trypsinogens 1 and 2 in two human pancreatic adenocarcinoma cell lines; CFPAC-1 and CAPAN-1

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Proteins with trypsin-like immunoreactivity (first detected by a specific immunoenzymatic assay) were isolated from CAPAN-1 and CFPAC-1 cell culture-conditioned media by chromatography on an immunoadsorbent prepared with a polyclonal antibody directed against trypsin 1. The adsorbed proteins were devoid of free trypsin activity but trypsin activity was present after enterokinase activation demonstrating that the immunoreactive trypsin present in cell supernatants corresponds to trypsinogens. When characterised by Western blotting using a monoclonal antibody directed against human trypsin 1 two protein bands corresponding to trypsinogen 1 (23 kDa) and trypsinogen 2 (25 kDa) gave a positive reaction. These results demonstrate the presence of trypsinogens 1 and 2 in CAPAN-1 and CFPAC-1 cells and in their culture-conditioned media.

Adenocarcinoma cell line; Pancreatic; Trypsinogen

1. INTRODUCTION

A new pancreatic adenocarcinoma cell line (CFPAC-1) was recently established from a patient with cystic fibrosis (CF) [1]. These cells express the CF gene and manifest the most common CF mutation/deletion of three nucleotides resulting in a Phe^{508} deletion (ΔF 508) [2]. As in other CF secretory epithelial cells, these cells are defective in cAMP-mediated activation of apical membrane channels, making this cell line useful in the further biochemical investigation of the CF defect. Since alteration in protein secretion has also been described in CF epithelial cells [3], we looked for protein markers secreted by CFPAC-1 cells preliminary to an investigation of protein secretion dysregulation in CF. Recent studies have shown that another established human pancreatic carcinoma cell line, CAPAN-1, contained a peptide similar or identical to pancreatic secretory inhibitor [4]. Because proteases and their inhibitors usually occur together, we looked for the presence of trypsin-like proteins in cell culture supernatants of CAPAN-1 and CFPAC-1. Our results, reported in this paper, demonstrate that CAPAN-1 and CFPAC-1, but not other tested pancreatic cell lines, secrete the two human pancreatic trypsinogens.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

2.1.1. CAPAN-1 and CFPAC-1

The CFPAC-1 pancreatic adenocarcinoma cell line (passage 26) from a patient with cystic fibrosis (CF) was cultivated under Schoumacher's conditions with some modifications. Cells were grown on type I collagen-coated flasks (Falcon) in IMDM (Iscove's modified Dulbecco's medium, Sigma) supplemented with 5% decomplemented fetal bovine serum (Eurobio) in the presence of 100 U/ml penicillin and 100 mg/ml streptomycin (Eurobio) and maintained at 37°C in a 5% CO_2/95% air atmosphere. Medium supernatants were removed every two days, collected and stored at −20°C until use. Cells were usually passaged every 8 days at 1:6 dilution by exposure to 0.05% trypsin in a 0.03% EDTA buffer at 37°C for 3 min. The human pancreatic adenocarcinoma cell line CAPAN-1 (passage 74) was cultivated as CFPAC-1 with the exception that the medium used was RPMI 1640 (Sigma) instead of IMDM. Media supernatants were collected as described above and stored at −20°C.

2.1.2. Other pancreatic adenocarcinoma cell lines

PANC-1, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in Dulbecco's MEM with 10% fetal bovine serum (FBS) at passage 72. CAPAN-2, obtained from the ATCC, was cultured in McCoy's 5A media with 10% FBS at passage 36. ASPC-1, obtained from the ATCC, was cultured in RPMI medium with 10% FBS at indeterminate passage, and CD 11, kindly provided by Dr. Richard Metzgar of Duke University [5], was cultured in MEM with 10% FBS at indeterminate passage. Medium supernatants were collected for two days and stored at −20°C before assays.

2.2. *DNA analysis of the CAPAN cell line with respect to the major CF mutation*

The genotype of CAPAN cell line with respect to the major CF mutation, (i.e. the Phe^{508} deletion), was analysed by the heteroduplex method [6]. PCR-amplified DNA fragments spanning the ΔF 508 position were generated using specific flanking primers (CF-16 B and

16 D) as described by Riordan et al. [2]. No heteroduplex, which types heterozygosy for the mutation, was observed upon gel electrophoresis. Mixing the amplified CAPAN fragments with a corresponding normal DNA fragment also did not result in heteroduplex formation, thus excluding a homozygous state for the ΔF 508 mutation. From these data we conclude that the ΔF 508 mutation is absent for both CFTR alleles of the CAPAN-1 cell line.

2.3. *Proteins and antibodies*

Human trypsinogens 1 and 2 were partially purified by chromatography of human pancreatic juice on DEAE-Trisacryl M at pH 8.0 in the presence of 1 mM benzamidine and lima bean trypsin inhibitor (5% of protein wt.) as previously described [7]. Trypsin 1 was prepared by autoactivation of partially purified trypsinogen 1 at pH 7.8 in the presence of 20 mM calcium, and purification by affinity chromatography on Sepharose coupled with aprotinin (Bayer AG) as previously described [8]. DFP-trypsin 1 was obtained by incubation of purified trypsin 1 with 10 mM diisopropylflurophosphate (DFP) 10^3-times molar excess) at pH 7.8 and 4°C for 24 h. Rabbit polyclonal antibodies directed against trypsin 1 were prepared by injection of purified trypsin 1. They recognized both trypsinogens 1 and 2 and their corresponding trypsins (data not shown) except in the immunoassay where only trypsin(ogen) 1 was measured. Monoclonal antibodies directed against trypsin 1 were prepared by immunisation of OF_1 mice with DFP-trypsin 1 and hybridization of spleen cells with myeloma cells in the conditions described elsewhere [9]. Clone G6, which recognizes the two trypsinogens and their corresponding trypsins, was used.

2.4. *Immunoenzymatic assays of pancreatic enzymes*

Human trypsin(ogen) 1 was measured by a non-competitive 'sandwich' enzyme immunoassay as described in detail elsewhere [10]. This immunoassay does not recognize human trypsinogen 2 or trypsin from other species. The presence of chymotrypsinogen and lipase in culture supernatants was assayed by specific non-competitive enzyme immunoassays [11,12].

2.5. *Gel filtration experiments*

The molecular size distribution of immunoreactive trypsin (IRT) present in culture supernatant was determined by a Sephadex G100 SF filtration in a 50 mM Tris-HCl buffer containing 200 mM NaCl at pH 7.6 and 4°C. The loaded sample was 2 ml and the collected fractions were 1 ml with an elution rate of 3 ml/h. IRT was measured in each fraction. Column calibration was made with the following reference proteins: cytochrome *c* (M_r 12 500) from Fluka; chymotrypsinogen A (25 000), ovalbumin (45 000) and bovine serum albumin (66 000) from Pharmacia Fine Chemicals.

2.6. *Immunoadsorbent chromatography*

Immunoglobulin G (IgG) was purified by precipitation of rabbit immunserum anti-trypsin 1 with caprylic acid according to the technique of Steinbuch and Audran [13]. IgG was fixed to activated Sepharose (41 mg of IgG per 2 g of gel) following the method of Cuatrecasas [14]. Before chromatography, 400 ml of cultured media were dialyzed overnight against 0.05% NH_4HCO_3 and concentrated by lyophilisation. Lyophilized culture media dissolved in water (15 ml of CFPAC-1 and 17 ml for CAPAN-1) were loaded on the immunoadsorbent column equilibrated in a 25 mM phosphate buffer, pH 7.0, containing 1 M NaCl. Adsorbed proteins were eluted by a 0.2 M glycine-HCl buffer at pH 2.5. The elution of immunoreactive trypsin was followed by enzyme immunoassay.

2.7. *Western blotting*

Chromatographic fractions were concentrated by TCA precipitation for electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [15] in non-reducing conditions, in a 7.5–15% acrylamide concentration-gradient slab gel. After transfer of proteins to nitrocellulose as described by Burnette [16], immobilized proteins were characterized by immunodetection using monoclonal antibody prepared against trypsin 1. The monoclonal antibody, which recognizes both human trypsinogens, was diluted (1:500) in phosphate buffered saline, pH 8.0, containing 10% new-born calf serum and 0.2% Triton X100. The immune complexes were detected by a color reaction using a second antibody conjugated to horseradish peroxidase and staining with diaminobenzidine in the presence of hydrogen peroxide, as described by Coudrier et al. [17].

2.8. *Activation of trypsinogen(s) by enterokinase*

Immunoadsorbent chromatographic fractions containing the highest concentrations of immunoreactive trypsin 1 were pooled and used for activation. Aliquots (0.6 ml) of trypsinogen solution (containing 72 ng and 257 ng of immunoreactive trypsin 1 for CAPAN-1 and CFPAC-1, respectively) were exposed to enterokinase (in excess amount) in a Tris-HCl buffer at pH 7.8 containing 10 mM $CaCl_2$ at 4°C. Samples of this activation mixture were removed periodically and trypsin activity was measured by a spectrofluorimetric method using *N*-α-benzyloxycarbonyl-L-arginine (4-methyl-7-coumaryl) amide (10 μM) as the enzyme substrate, as described elsewhere [18].

2.9. *Preparation of cell lysates*

Cells were rinsed three times with a PBS buffer (Dulbecco A) and then incubated in a 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl and 10 mM EDTA. The cells were scraped off and sonicated three times for 5 s at 4°C. Protein concentration was measured according to Bradford [19].

3. RESULTS

3.1. *Demonstration of immunoreactive trypsin 1 in CAPAN-1 and CFPAC-1 cell lines*

The presence of pancreatic enzymes was studied in the supernatants of 6 cell lines, PANC-1, CAPAN-1, CAPAN-2, CFPAC-1, ASPC-1 and CD-11. No immunoreactive lipase and chymotrypsinogen was detected in any cell line. Only immunoreactive trypsin 1 was found in CAPAN-1 and CFPAC-1 supernatants at the level of 10 ng/ml. In the homogenates of the same cell lines performed at confluency (approximately 5×10^6 cells/25 cm^2) immunoreactive trypsin 1 concentration was 4 ± 2 ng/mg of protein in both cell lines.

3.2. *The molecular size distribution of immunoreactive trypsin in cell culture supernatant*

The molecular size distribution of immunoreactive trypsin in cell culture supernatant was determined by immunoassay of trypsin in the different fractions obtained by gel filtration. Identical profiles were observed for CAPAN-1 and CFPAC-1 supernatants and a typical profile is presented in Fig. 1. Fractions containing immunoreactive trypsin 1 were eluted at the end of the second void volume with the proteins of 25 kDa, well separated from the general protein peak. This elution profile is consistent with the presence of a form of inactive trypsin, probably trypsinogen, in the supernatant. Active trypsin should be eluted in the first chromatographic fractions as complexes associated with serum inhibitors.

3.3. *Isolation of trypsin-like proteins from CAPAN-1 and CFPAC-1 cell culture supernatant by immunoadsorbent chromatography*

Trypsin-like proteins were isolated by immunoad-

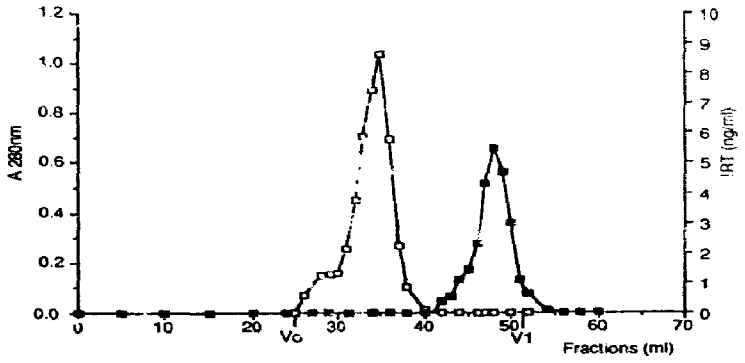

Fig.1. Identification of the molecular forms of immunoreactive trypsin (IRT) by gel filtration of cell lines' supernatant on Sephadex G 100 SF. Column 1.5 × 80 cm; load, 2 ml of supernatant; fraction volume, 1 ml. *A* 280 nm (□), IRT (■).

sorbent chromatography on an affinity-column of anti-trypsin 1. After dialysis and concentration by lyophilisation, culture media were dissolved in water and loaded on the immunoadsorbent column. Adsorbed proteins were eluted by acidic pH and immediately neutralized at pH 6.0 by the addition of convenient amounts of a Tris-HCl buffer at pH 8.0. The elution was followed by immunoenzymatic assay which recognized only trypsin(ogen) 1, and the fractions containing immunoreactive trypsin were further analysed by Western blotting.

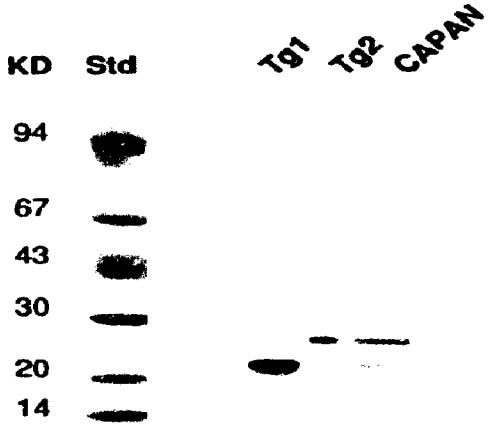

Fig. 2. Western blots of trypsinogens isolated from CAPAN-1 cell culture medium. Reference proteins (Std) used were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean inhibitor (20 kDa) and α-lactalbumin (14 kDa). Std proteins were stained with Ponceau S; trypsinogens (Tg) were immunodetected in CAPAN, and samples of Tg1 and Tg2, with a monoclonal antibody to trypsin 1.

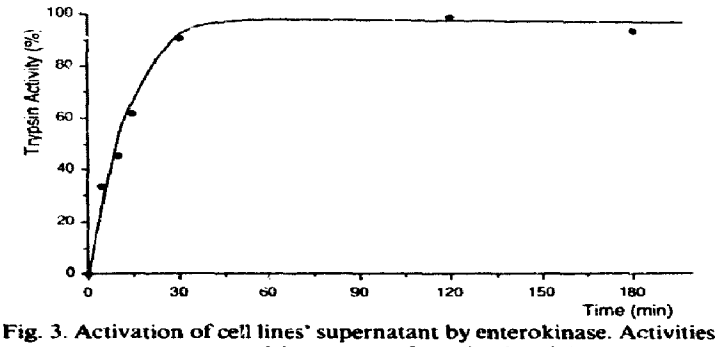

Fig. 3. Activation of cell lines' supernatant by enterokinase. Activities were expressed in percent of maximal activity.

3.4. *Characterization of immunoreactive trypsin contained in CAPAN-1 and CFPAC-1 cell culture supernatant*

The composition of trypsin-like proteins present in cell supernatants and adsorbed on anti-trypsin immunoadsorbent was determined by Western blotting. The results of the immunodetection with the monoclonal antibody G6, which recognizes both human trypsin(ogen)s, are shown in Fig. 2. Two positive reactions are present on immunoreactive trypsin-concentrated fractions isolated from CAPAN-1 supernatant. The protein bands correspond to the mol. wts. of 23 and 25 kDa and are consistent with the mol. wts. of, respectively, trypsinogens 1 and 2 purified from human pancreatic juice [20]. The same results were obtained with immunoreactive trypsin-concentrated fractions isolated from CFPAC-1 supernatant.

3.5. *Enterokinase activation of trypsin-like proteins*

The fractions with the highest immunoreactive trypsin levels obtained by immunoaffinity chromatography of CAPAN-1 and CFPAC-1 cell culture-conditioned media were pooled and assayed for trypsin activity. These fractions were devoid of trypsin activity until activated by enterokinase. The kinetics of activation of CAPAN-1 trypsinogen is shown on Fig. 3. Trypsin activity reaches a plateau after 30 min and remains stable for 3 h at 0°C. The same activation was obtained with CFPAC-1 trypsinogen. This experiment demonstrates conclusively that immunoreactive trypsin present in CAPAN-1 and CFPAC-1 is in the form of trypsinogens.

4. DISCUSSION

In this study, we demonstrate the expression of the two human pancreatic trypsinogens in two human pancreatic cell lines CFPAC-1 and CAPAN-1. The normal pancreatic gland is composed of numerous exocrine and endocrine cell types which are believed to originate from an endodermic stem cell [21]. These different cell types do not seem to have the same ability to develop tumors. The majority (over 80%) of cancers of

exocrine pancreas appear to arise from ductal epithelium [22]. All pancreatic cell lines studied in this report have been described as deriving from tumors of pancreatic ducts. From all pancreatic cell lines examined here, only CAPAN-1 and CFPAC-1 were shown to secrete trypsinogens. Since the CFPAC-1 cell line has been established from a patient with cystic fibrosis homozygous for the common CF mutation, we first excluded the possibility that CAPAN-1 could unexpectedly present the same genotype. The presence of other CF mutations on CAPAN-1 cells is not totally excluded but it seems unlikely that a CF mutation was responsible for the presence of trypsinogens. The possibility that the CAPAN-1 and CFPAC-1 cell lines contain some acinar cells responsible for trypsinogen synthesis and secretion also seems to be excluded, since other pancreatic enzymes well represented in human external secretion, such as chymotrypsinogen and lipase, have not been detected in culture supernatant by sensitive immunoenzymatic assays, even after a 10-fold concentration.

Different possibilities can be suggested to explain our findings. Neutral proteases, mainly plasminogen activator [23] and collagenases [24], have long been thought to be involved in the degradation of extracellular matrix and tumor invasion. The recent demonstration of the expression of the two human trypsinogens in cyst fluid of mucinous ovarian tumors indicate a possible role of these proteins in the protease cascade in tumor invasion [25,26]. In this respect it is interesting to note that the only two cell lines producing trypsinogens were both established from liver metastasis [1,27]. On the other hand, unlike the other pancreatic cell lines, both CAPAN-1 and CFPAC-1 cells spontaneously formed duct-like structures in the stationary phase of growth. Electron micrographs show apical microvilli and glycocalyx, subapical cytoplasmic vacuoles containing mucin granules and tight junctions between adjacent cells [1,28]. During differentiation in culture, CAPAN-1 cells were shown to form domes which are thought to be the morphological expression of transepithelial transport of water and electrolytes [29]. We observed a similar dome formation of CFPAC-1 cells in culture (unpublished data). These morphological modifications suggest a possible relationship between the presence of trypsinogens and a peculiar stage of pancreatic ductal cell differentiation. Since recent histological findings have shown that the intra- and interlobular duct cells of organ cultures of human fetal pancreas stain positively for trypsinogen (B. Tuch, personal communication), the possibility that the two neoplastic cel lines, CAPAN-1 and CFPAC-1, have regressed back to a fetal form, a feature of so many neoplasms, is therefore worth considering.

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