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Quantification of pancreatic secretory trypsin inhibitor in colonic carcinoma and normal adjacent colonic mucosa

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Abstract

Aims: **To measure the content of immunoreactive human pancreatic secretory trypsin inhibitor (irPSTI) in colonic carcinoma and adjacent normal colonic mucosa.**

Methods: **From a stable hybridoma cell line producing monoclonal antibodies specific for human PSTI, a specific enzyme linked immunosorbent assay (ELISA) for human PSTI was developed. In a precipitation assay system these antibodies bound human PSTI in a dose-dependent manner. The specimens were obtained from resectional surgery.**

Results: **The content of irPSTI was 19·9 μg/g protein (0·55 μg/g tissue wet weight) in colonic carcinoma. In adjacent normal colonic mucosa 43·6 μg/g protein (1·12 μg/g tissue wet weight) was shown.**

Conclusions: **The enzymatic degradation of surrounding tissue necessary for tumour cell invasion could be facilitated by this relative deficit of the inhibitor in infiltrative carcinoma.**

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Pancreatic secretory trypsin inhibitor (PSTI) was first isolated from pancreatic tissue in 1948 by Kazal.[1] PSTI is thought to protect the pancreatic gland from damage by preactivated trypsin. The serum concentration of immunoreactive PSTI (irPSTI) is increased in severe diseases such as carcinoma of pancreatic gland, breast, thyroid, stomach, oesophagus,[2] and gall bladder.[3]

irPSTI has also been shown in and isolated from different parts of the normal gastrointestinal mucosa.[4 10] irPSTI has been found in the foveolar cells in the gastric mucosa. In duodenal and small intestinal mucosa irPSTI found in Paneth[5 7] and goblet cells.[5 8] Normal gall bladder mucosa did not contain irPSTI while well differentiated carcinoma and tissue resembling adenoma did.[11]

In colonic mucosa ir PSTI has been found in the goblet cells in the basal parts of the crypts.[6] In colonic adenomas, however, irPSTI was also found in the upper parts of the polyps following the reverse mode of regeneration.[12] irPSTI has not been shown in most colonic carcinoma tissue.[13]

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Methods

Electrophoresis reagents, dextran T500, protein A sepharose Cl-4B, and CNBr-activated sepharose CI-4B were produced by Pharmacia LKB Biotechnology, Uppsala, Sweden. Class specific goat anti-mouse immunoglobulins were obtained from Nordic Immunologic Laboratories, Tilburg, the Netherlands. Alkaline phosphatase conjugated avidin was obtained from Dakopotts AB, Hägersten, Sweden. Tissue culture medium and fetal calf serum were supplied by Gibco Laboratories, Grand Island, New York, USA. Pansorbin was obtained from Calciobiochem Corporation, San Diego, California, USA. Recombinant PSTI (rPSTI) was obtained from Synergen Inc, Boulder, Colorado, USA. All other chemicals and proteins were purchased from Sigma Chemical Co, St Louis, Missouri, USA.

PRODUCTION OF MONOCLONAL ANTIBODIES

BALB/c mice (Bommice, Bomholtgärd Breeding and Research Center Ltd, Bomholtvej, Denmark) were immunised by a subcutaneous injection of 30 μg of rPSTI in complete Freund's adjuvant, repeated five times at three week intervals with 30 μg rPSTI in Freund's incomplete adjuvant. Hybridomas were produced by fusion of SP2/0, non-secreting BALB/c myeloma cells with spleen lymphocytes from two out of five immunised mice.[14 15]

SELCTION OF ANTIBODY-PRODUCING HYBRIDOMAS

Hybridoma culture fluids were tested for antibody using solid phase ELISA technique. Microtitre plates were coated with 50 μl of rPSTI, 10 μg/ml. Hybridomas producing antibodies specific for PSTI were selected for cloning. The subclass type of the monoclonal antibodies produced was determined by the double immunodiffusion test.[16] Cells harvested from cultures of hybridomas producing monoclonal antibodies against PSTI were injected intraperitoneally into BALB/c mice treated with Pristane (Aldrich, Beerse, Belgium) for the production of ascites fluid. The ascites fluid was purified by affinity chromatography on a protein A sepharose CI-4B column equilibrated with 1·5 M glycine-NaOH, 3 M NaCI, at pH 8·9. The column was washed with this buffer after application of the ascites fluid and the IgG was eluted stepwise with 0·1 M citric acid-NaOH at pH

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6·0, pH 5·0, pH 4·0 and pH 3·0. IgG1 was eluted at pH 6·0. This IgG1 fraction was loaded on a CNBr-activated sepharose CI-4B column covalently attached with 70 mg rPSTI. Non-specific binding protein was eliminated by rinsing the column with the TRIS-HCl buffer and specific elution of the PSTI antibodies was accomplished using reversed flow elution with 0·1 M glycine/HCl, 3M NaCI, at pH 3·0.

RABBIT ANTISERUM AGAINST HUMAN PSTI

A polyclonal antiserum against human PSTI was obtained by immunising rabbits with a subcutaneous injection of 200 μg rPSTI in Freund's complete adjuvant, repeated three times at three week intervals with 200 μg rPSTI in Freund's incomplete adjuvant. The production of mono- and polyclonal antibodies was approved by the ethical committee for animal experiments at the University of Lund.

BIOTINYLATION OF RABBIT ANTI-HUMAN PSTI ANTIBODIES

IgG fractioned rabbit anti-human PSTI antibodies (5 mg) obtained after separation on a protein A sepharose CI-4B column, in 1 ml glycine/HCI buffer, were dialysed against 0·1 M $NaHCO_3$ overnight at +2°C. N-hydroxysuccinimidobiotin (120 μl 1 mg/ml) in dimethyl sulfoxide was added to the antibody solution and incubated for four hours at room temperature. The biotin labelled antibodies were then dialysed against 0·01 M phosphate buffered saline (PBS), pH 7·2, at +4°C, overnight.

INDIRECT IMMUNOPRECIPITATION OF PSTI BY MONOCLONAL ANTIBODIES

Human PSTI (2 μg) was incubated in a total volume of 100 μl with an increasing amount of monoclonal antibody (2 mg/ml) and bovine serum albumin (to equalise the protein content in each tube (5 μg/100 μl) in 0·05 M TRIS-HCI buffer, 0·005 M EDTA, at pH 7·4, at room temperature for 30 minutes. The reaction mixture was further incubated at room temperature for 30 minutes after the addition of 10 μl of 20 μg/ml rabbit anti-mouse IgG and at +4°C for 30 minutes after the addition of 60 μl of Pansorbin suspension. The immune complexes were precipitated by centrifugation at +4°C. The resulting supernatant fluids (100 μl) were taken for the measurement of trypsin inhibition after adding 600 μl 0·05 M TRIS/HCl, CaCl 0·02 M, pH 8·2, and 100 μl trypsin 1 μg/ml with N-benzoyl-isoleucine-glutamine-glycine-arginine p-nitro-anilide hydrochloride as a substrate. Absorbance was measured at 405 nm. The control experiments were carried out with normal mouse IgG instead of monoclonal antibody in the initial step of the incubation.

ELISA

Carbonate buffer, pH 9·6 (0·015 M Na_2CO_3 and 0·035 M $NaHCO_3$), was used as coating buffer for the monoclonal PSTI antibody. 0·05 M TRIS–HCl buffer, pH 7·4, containing 0·05 M $CaCl_2$, 0·5 M NaCl, and 0·2% bovine serum albumin was used as sample dilution buffer. PBS containing 0·05% Tween-20, pH 7·4, was used for washing. Between each incubation step the wells were washed five times with PBS-Tween. The wells were coated with 100 μl monoclonal PSTI antibody, 15·0 mg/l for 24 hours at +4°C.

Sample buffer (100 μl) containing 1% bovine serum albumin in each well was incubated for at least two hours at room temperature. Then 100 μl of diluted sample or standard were applied per well followed by overnight incubation at +4°C. The test samples were used in serial dilutions.

The next incubation step involved 100 μl of biotin conjugated rabbit-anti-PSTI antibodies (5 mg/ml), diluted 1 in 600 in the sample buffer, for two hours at room temperature. After washing, alkaline phosphatase conjugated avidin 1 in 1000 was added to the samples. After a final wash 100 μl of phosphatase substrate in 10% ethanolamine buffer, pH 9·8, containing $MgCI_2$ (10 mmol/l) were applied and incubated at +25°C. The plates were then analysed in an automatic Titertek multiscan photometer at 405 nm.

TISSUE SPECIMENS

Specimens were obtained from 12 consecutive patients who had undergone colonic resection because of carcinoma (one patient had two

Contents of irPSTI in normal colonic mucosa and colonic carcinoma

µg irPSTI/g protein		*µg irPSTI/g tissue wet weight*		
Normal mucosa	*Cancer*	*Normal mucosa*	*Cancer*	*Differentiation of the adenocarcinoma*
75·5	22·5	1·79	0·66	Well to moderate
31·5	15·7	0·93	0·38	Well to moderate, mucus producing
59·0*	14·5	1·59*	0·44	Well to moderate, highly mucus producing
59·0*	11·1	1·59*	0·30	Moderate, mucus producing
47·1	7·9	1·19	0·20	Moderate
23·9	12·5	0·70	0·37	Moderate, poorly mucus producing
16·1	14·5	0·37	0·26	Moderate to poor, poorly mucus producing
46·5	24·2	1·25	0·70	Moderate
70·8	25·1	1·97	0·59	Moderate, partly mucus producing
32·8	29·5	0·99	0·64	Moderate
65·4	41·4	1·88	1·38	Moderate to poor, partly mucus producing
22·5	7·8	0·66	0·27	Moderate to poor
32·4	31·4	0·82	0·98	Partly poor, partly mucus producing

*One patient had two carcinomas.

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colonic carcinomas). Clinical examination of the patients preoperatively did not reveal any other serious disease. From each resected colonic specimen one piece was taken from the carcinoma and one piece from the normal mucosa. The specimens were frozen and stored at −20°C for later analysis. The specimens were thawed and homogenised in four parts 0·05 mM acetic acid with aprotinin (Trasylol) 500 KIU/ml. The homogenates were freeze-thawed five times. The homogenates were centrifuged 15000 × *g* for 10 minutes. The supernatants were collected. The precipitates were suspended in two parts acetic acid twice and centrifuged after each suspension. The three supernatant fluids were pooled and stored at −20°C until analysed with the above described ELISA and protein concentration by a modification of Lowry's method.[17]

Histological evaluation was done according to WHO tumour classification and grading.[18]

The Wilcoxon matched-pairs signed ranks test was used for statistical analysis.

Results

The immunoprecipitation of PSTI by monoclonal antibodies showed a dose dependent binding of PSTI to the antibody. In the ELISA the intra-assay variation was 7% and the interassay variation was 13%. The lower limit of detection was 1 μg/l. The same recovery was obtained for PSTI in the tissue samples after dilution as after dilution of pure human PSTI (parallel dilution curves).

The content of irPSTI in the specimens stated as μg/g protein and μg/g tissue wet weight is listed in the table. In all patients malignant tissue contained less irPSTI than the normal adjacent mucosa. The median content in carcinoma was 19·9 μg/g protein, range 7·8–41·4 (0·55 μg/g tissue wet weight, range 0·20–1·38). The content in normal colonic mucosa was 43·6 μg/g protein, range 16·1–75·5 (1·12 μg/g tissue wet weight, range 0·37–1·97). This difference was significant ($p < 0·01$).

Discussion

PSTI isolated from gastric, small intestinal, and colonic mucosa has been shown to be identical with that from pancreatic gland.[6 7 9] In this study a specific monoclonal antibody against human PSTI was produced. With these antibodies an ELISA was developed for human PSTI. Analyses with this method in 12 patients with 13 colonic carcinomas showed that the median content of PSTI was lower in malignant than in normal colonic mucosa. Although there was an overlap in the range of tissue values, we universally found a lower PSTI content in malignant rather than in normal adjacent mucosa. These findings agree with our earlier findings of immunohistochemical staining results on specimens containing normal colonic mucosa, adenomas, and colonic carcinomas.[4 13] In those studies irPSTI was shown in the basal parts of the crypts in normal colonic mucosa but rarely in colonic carcinomas. In polyps containing carcinomatous changes without infiltration of the muscularis mucosa, we noticed a shift from a high PSTI content in adenomatous epithelium to the carcinomatous epithelium where irPSTI was absent.[13] This shift in content of irPSTI is difficult to explain. The apparent lack of the inhibitor could, however, be explained by increased consumption, because there is evidence to suggest that proteinase production is necessary for tumour cell invasion and that this proteolytic reaction takes place close to the tumour cell.[19 22] Recently two tumour associated trypsin(ogen)s were detected in ovarian carcinoma. These proteases were expressed together with tumour associated trypsin inhibitor (TATI).[23] TATI is probably identical with PSTI.[24] The explanation of increased consumption also agrees with the recently demonstrated PSTI mRNA in colorectal cancer.[25] Gastrointestinal PSTI in normal and neoplastic mucosa may provide an important and hitherto unrecognised protective mechanism. Further studies are required to determine factors which control the secretion of gastrointestinal PSTI and its possible role in gastrointestinal mucosa and carcinoma.

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