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Co-expression of trypsin and tumour-associated trypsin inhibitor (TATI) in colorectal adenocarcinomas

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Summary. Trypsin and its specific inhibitor, TATI (tumour-associated trypsin inhibitor), are expressed in normal human pancreas and in a variety of tumours. The aim of the present study was to assess the parallel expression of trypsin and TATI in colorectal cancer, in comparison with their expression in normal epithelial tissue, since proteases and their inhibitors are thought to be co-expressed in malignant neoplasms. We also assessed the possible significance of their expression as a means of differentiation between normal and malignant tissue. We examined qualitatively and semi-quantitatively the immunohistochemical expression of trypsin and TATI on paraffin-embedded serial tissue sections from 91 colorectal adenocarcinomas. The reverse-transcriptase-polymerase-chain reaction (RT-PCR) was also performed on fresh malignant tissue from 55 of the above adenocarcinomas. Normal and non-malignant tissues adjacent to the tumours were also evaluated. Cytoplasmic expression of trypsin (more than 25% of the cancer cells positive) was found in 67 (73.6%) adenocarcinomas, whereas TATI was expressed in the cytoplasm of 59 (64.8%) cases studied. Statistical analysis using Spearman's test has demonstrated a significant correlation between trypsin and TATI immunohistochemical expression ($p<0.01$). RT-PCR showed co-expression of trypsin and TATI mRNA in all carcinomas studied. Distinct patterns of trypsin and TATI immunohistochemical expression were observed in adjacent, non-malignant tissues, where both trypsin and TATI mRNA were also detected. Normal tissues were negative by immunohistochemistry. Our results indicate co-expression of trypsin and TATI in colorectal tumours both at the mRNA and protein level. We conclude that in colorectal neoplasms, high levels of trypsin and TATI may be important for malignant tumour formation and/or metastatic process.

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Introduction

Proteases and their inhibitors usually occur together in tissues and are thought to contribute actively in tumour invasion and metastasis (Verspaget, 1998). Trypsin is one of the best characterized serine proteases. It is produced as a zymogen (trypsinogen) by the acinar cells of the pancreas, secreted into the duodenum, and activated into the mature form by enterokinase, which functions as an essential food-digestive enzyme. Past studies have shown that trypsin is also expressed by normal bronchial, bronchiolar and alveolar epithelial cells, esophagus, stomach, small intestine, colon, epithelia of intrahepatic large ducts, septal ducts and peribiliary glands, liver and extrahepatic bile duct, splenic cells, skin, vascular endothelial cells and neuronal cells (Terada and Nakanuma, 1991; Kawano et al., 1997; Koshikawa et al., 1997, 1998; Terada et al., 1997). Trypsin has also been detected in human cancer cells of the stomach (Terada et al., 1997), ovary (Hirahara et al., 1995), lung (Kawano et al., 1997), colon (Terada et al., 1997; Bernard-Perrone et al., 1998) and the human male genital tract (Paju et al., 2000).

Two trypsinogen isoenzymes are produced by ovarian tumours. They are purified from cyst fluid of mucinous ovarian adenocarcinoma and are similar to pancreatic trypsinogen-1 and -2 with respect to the amino-terminal sequence, molecular weight and immunoreactivity, but show different isoelectric points and stability. Hence, they have been named Tumour-associated trypsinogen-1 and -2 (TAT-1 and TAT-2), respectively (Koivunen et al., 1989).

TAT-1 and -2 are thought to be the target enzymes of Tumour-associated trypsin inhibitor (TATI), a 6 kDa peptide initially detected in the urine of patients with

ovarian cancer (Stenman et al., 1982). TATI is identical to Pancreatic secretory trypsin inhibitor (PSTI) and has a well-established function as an inhibitor of trypsin (Fritz et al., 1967) and acrosin (Huhtala, 1984). Elevated TATI concentrations have been observed in the serum of patients with various cancer types. TATI is expressed by the acinar cells of pancreas and also by cancer cells of colon (Higashiyama et al., 1990a), stomach (Higashiyama et al., 1990b), lungs (Higashiyama et al., 1992), ovary (Ueda et al., 1989), gallbladder (Bohe et al., 1991), liver (Ohmachi et al., 1993) and kidney (Lukkonen et al., 1999) and by various cell lines (Koivunen et al., 1991). It is also produced by the mucosal cells of the gastrointestinal tract, where it is thought to protect these cells from proteolytic breakdown (Freeman et al., 1990a).

The aim of our study was to assess the parallel expression of trypsin and TATI in colorectal adenocarcinoma specimens, both at the mRNA and the protein level, in comparison with their expression in normal epithelial tissue. We also assessed the possible significance of their expression as a means of differentiation between normal and malignant tissue. To our knowledge this is the first attempt of parallel study of trypsin and TATI in colorectal adenocarcinoma.

Materials and methods

Patients, tissues and cell culture

Tissue samples were obtained from patients that underwent surgery at the Hippokration General Hospital of Athens, Greece, between 1997 and 1998. The consent of patients was in all cases obtained. Formalin-fixed, paraffin-embedded tissues were obtained from 91 colorectal cancer patients, 53 males and 38 females, aged 50-91 years (mean age = 69 years). 51 patients had colon cancer and in 40 patients, tumours were confined in the rectum. Seventy three tumours were histologically classified as adenocarcinomas and 18 as mucinous adenocarcinomas, whereas 40 were low-grade and 51 high-grade adenocarcinomas. Fourteen neoplasms were staged as Dukes' A, 30 as Dukes' B, 33 as Dukes' C and 14 as Dukes' D. Fresh tissues, immediately frozen in liquid nitrogen and stored at -80 °C until use, were obtained from those of the above patients (55) that had a surgically resectable tumour. Sampling was performed by an experienced pathologist, so as to minimize the possibility of the presence of adjacent, non-malignant cells.

As normal controls, we used paraffin-embedded tissues from 17 individuals (9 males and 8 females, aged 30-78 years, mean age = 60 years) that underwent colonoscopy and biopsy, and histological examination showed non-specific infection of the mucosa, or no lesions at all. We also used fresh, presumably non-malignant tissue obtained, only in cases when it was possible, from a distance more than 10 cm from 9 of the adenocarcinomas studied.

As positive controls, we used paraffin-embedded, normal pancreatic tissue, adjacent to adenocarcinoma of a patient with cancer of the head of pancreas, and fresh normal pancreatic tissue obtained during an operation from a patient with MALT-lymphoma of the stomach. We also used the human colon adenocarcinoma cell line COLO 205 (ECACC, CAMR, Salisbury, UK), which is known to produce TATI and Tumour-associated trypsinogen (Koivunen et al., 1991).

Cell culture was maintained in RPMI 1640 medium with Glutamax I (Gibco BRL, UK) supplemented with 10% fetal bovine serum and 1% HEPES buffer 1M (Biochrom AG Seromed, Germany). The cells were grown at 37 °C in a humidified atmosphere with 5% CO2. Cells were expanded to 75-cm^2 cell-culture flasks and pellets containing 10^7 cells were stored at -80 °C until use.

Immunohistochemistry

Specific monoclonal antibodies against trypsin-1 (Clone MAB1482) and TATI (Clone 11B3) were purchased from Chemicon International Inc, CA and Orion Diagnostica, Finland, respectively. The dilutions of antibodies used were standardised at 1:300 and 1:40 in PBS buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.5% serum from a non-immunized animal (Dako Corp., Denmark) for trypsin and TATI, respectively.

For immunohistochemical staining we used a modification of the immunoglobulin enzyme bridge technique (avidin-biotin-peroxidase, ABC, method) reported by Hsu et al (1981). Paraffin-embedded serial tissue sections of 5 μm were first dried for 30 min at 58 ºC before deparaffinization in xylene and rehydration by sequential incubation in ethanol/water solutions. The sections were treated with 3% hydrogen peroxide in methanol, for 30 min at room temperature, in darkness, to quench endogenous peroxidase activity. After rinsing in water and PBS, sections were blocked with serum from a non-immunized animal (1:5 in PBS) for 20 min, to reduce non-specific binding, and were subsequently incubated with primary monoclonal antibody in a moist chamber at 4 °C, overnight. After washing in PBS, biotinylated anti-mouse immunoglobulin (Dako Corp., Denmark) at a dilution of 1:200 was added for 45 min, followed by a washing step and incubation with ABC reagent (streptABComplex/HRP, Dako Corp., Denmark) for 45 min. Peroxidase reaction was developed with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) in PBS buffer containing 0.06% hydrogen peroxide, for 1 min (trypsin staining) or 3 min (TATI staining). Finally, sections were rinsed with water and counterstained with Harris' Hematoxylin. The degree of immunoreactivity was assessed independently by two viewers (SS and KP), as the percentage of positive cancer cells in ten optical fields per section. Cases were considered as negative for trypsin or TATI expression when less than 5% of the cells were immunostained. There was no significant inter-observer variation.

As positive control, normal pancreatic tissue was stained. Negative controls, in which serum from a non-immunized animal had replaced primary antibody, were also included.

RT-PCR

Total RNA was extracted from fresh tissue specimens or exponentially growing cultured cells, by using the RNeasy Mini Kit (Qiagen Ltd, UK), according to the manufacturer's instructions.

One μg of total RNA was reverse transcribed into cDNA using Oligo (dT)15 primer and M-MLV reverse transcriptase, RNase H minus (Promega Corp., Madison), according to the manufacturer's instructions.

PCR Oligonucleotide primers for trypsin and TATI were constructed, as described previously (Bernard-Perrone et al., 1998; Lukkonen et al., 1999). The pair of primers for trypsinogen 1 produced a fragment of 379 bp and the pair of primers for TATI produced a fragment of 243 bp.

Reverse transcription product (5 μl) was amplified in a 50 μl reaction in 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmoles of each primer and 2.5 units of Taq DNA polymerase (Promega Corp., Madison). For trypsinogen 1 fragment amplification (Bernard-Perrone et al., 1998), samples were initially denatured for 1 min at 95 ºC. Cycling parameters (35 cycles) were: denaturation for 1 min at 95 ºC; annealing for 1 min at 69 °C; and extension for 1 min at 72 ºC. A final extension of 5 min at 72 ºC was performed. For TATI fragment amplification (Lukkonen et al., 1999), samples were initially denatured for 3 min at 94 ºC. Cycling parameters (30 cycles) were: denaturation for 1 min at 94 ºC; annealing for 1 min at 53 ºC; and extension for 30 sec at 72 ºC. A final extension of 10 min at 72 ºC was performed. Negative control experiments were performed, without the use of target RNA.

Portions of the amplified products were analyzed by agarose gel electrophoresis (1.5%) in the presence of ethidium bromide.

Statistical analysis

Kruskal Wallis and Mann-Whitney non-parametric tests were used to compare the immunohistochemical expression of trypsin or TATI among groups with different clinicopathological characteristics. Multiple regression analysis (parameters: sex, age, tumour location, Dukes' stage, histological type, grade) was also performed. Spearman's test was employed to evaluate the correlation between trypsin and TATI immunohistochemical staining (percentage of positive cancer cells). A p value less than 0.05 was considered statistically significant. Statistical analyses were performed with the SPSS 8.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Qualitative evaluation of immunohistochemistry

In normal pancreatic tissue, trypsin and TATI were

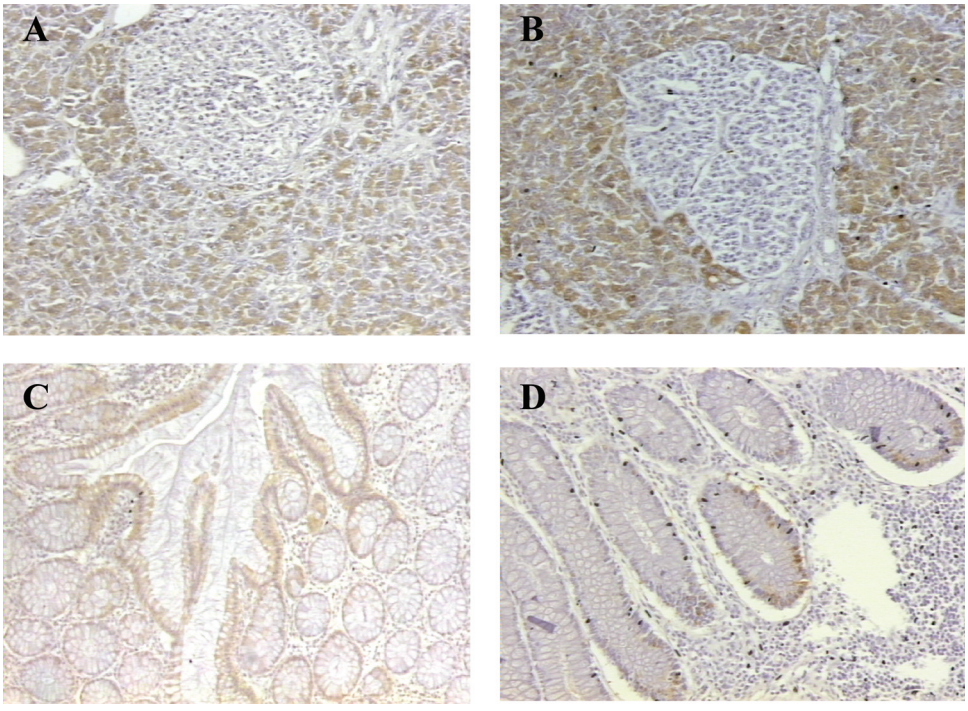

Fig. 1. Immunoexpression of trypsin and TATI in non-malignant tissues. Trypsin **(A)** TATI **(B)** in the cytoplasm of acinar cells of normal pancreas. Langerhans islets remain unstained. Trypsin **(C)** and TATI **(D)** in non-malignant tissue adjacent to colonic adenocarcinoma. Trypsin is expressed by the surface epithelium, while TATI is confined to the bsal crypts of the mucosa. A-C, x 10; D, x 40

selectively detected in the cytoplasm of acinar cells, while Langerhans islets (that do not produce trypsin or TATI) remained unstained (Fig. 1A,B). Sections from normal biopsies were negative for both trypsin and TATI, but non-malignant mucosa, adjacent to adenocarcinomas, showed distinct patterns of immunoexpression of the two antigens. Trypsin was detected in the surface epithelium (Fig. 1C), while TATI was confined to the basal crypts of the mucosa (Fig. 1D). The rest of the non-malignant mucosa remained unstained.

Cytoplasmic immunoexpression of trypsin and TATI was observed in a colon adenoma adjacent to colon adenocarcinoma (Fig. 2A) and in 67 (73.6%) and 59 (64.8%) out of 91 colorectal adenocarcinomas, respectively. Trypsin staining was diffuse in the

Table 1. Relationship between trypsin expression and clinicopathological parameters.

	TRYPSIN EXPRESSION (%)				
	<5	5-25	25-50	51-75	76-100
Tumour location					
Colon	1	10	9	10	21
Rectum		14	5	6	15
Dukes' Stage					
A		5	1	3	5
B	1	9	1	8	11
C		6	8	4	15
D		4	4	1	5
Histological type					
Adenocarcinoma	1	19	11	14	28
Mucinous adenocarcinoma		5	3	2	8
Grade					
Low		8	7	6	19
High	1	16	7	10	17
Total number of adenocarcinomas	1	24	14	16	36

Table 2. Relationship between TATI expression and clinicopathological parameters.

	TRYPSIN EXPRESSION (%)				
	<5	5-25	25-50	51-75	76-100
Tumour location					
Colon	5	12	15	10	9
Rectum	8	7	11	7	7
Dukes' Stage					
A		4	3	4	3
B	7	4	7	7	5
C	6	7	11	4	5
D		4	5	2	3
Histological type					
Adenocarcinoma	12	13	20	14	14
Mucinous adenocarcinoma	1	6	6	3	2
Grade					
Low	8	9	11	6	6
High	5	10	15	11	10
Total number of adenocarcinomas	13	19	26	17	16

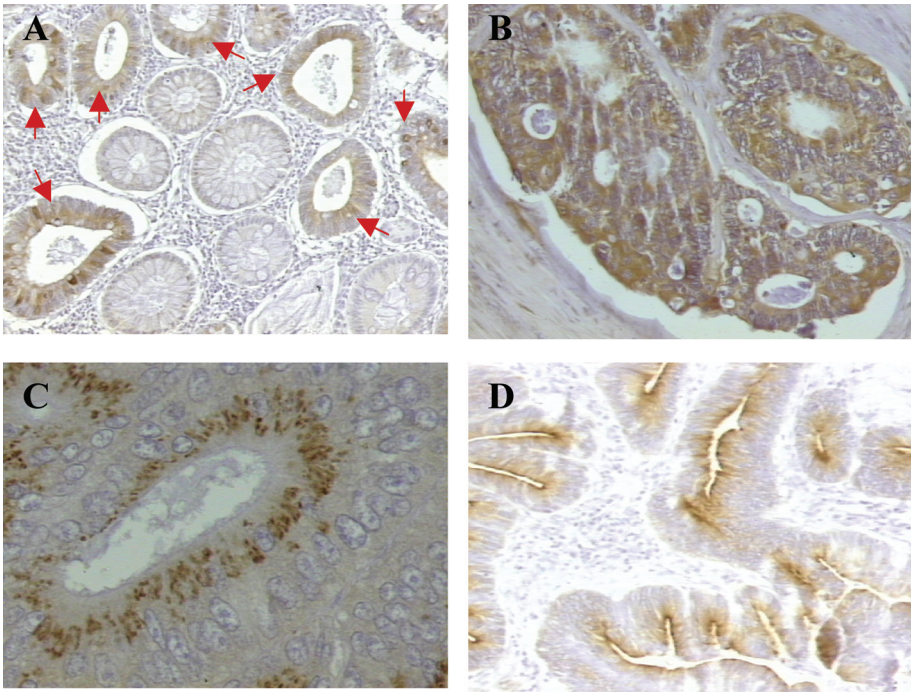

Fig. 2. Immunoexpression of trypsin and TATI in colorectal neoplasms. TATI in adenoma with severe epithelial dysplasia (arrows), while non-dysplastic crypts remain unstained **(A)**. Trypsin in adenocarcinoma Duke's C **(B)** and spotty pattern of trypsin in adenocarcinoma Duke's B **(C)**. TATI in adenocarcinoma Dukes' B **(D)**. A, x 10; B-D, x 40

cytoplasm of tumour cells (Fig. 2B), but in some cases a spotty pattern was observed (Fig. 2C). TATI staining was found mainly in the apical site of cancer cells (Fig. 2D). The intensity of trypsin and TATI expression was heterogeneous in colorectal neoplasms.

Semi-quantitative evaluation of immunohistochemistry

In Figure 3 the relationship between trypsin, or TATI immunoexpression (percentage of positive cancer cells) and clinicopathological parameters is presented. Kruskal-Wallis and Mann-Whitney tests showed no statistically significant difference between immunoexpression of trypsin or TATI among groups with different clinicopathological characteristics, stated in Figure 3. Multiple regression analysis has also failed to show a statistically significant difference of immunoexpression. Spearman's test showed a statistically significant correlation ($p<0.0001$, $r=0.398$) between the percentages of cancer cells found positive for trypsin and for TATI in serial sections of individual colorectal adenocarcinomas (Fig. 4).

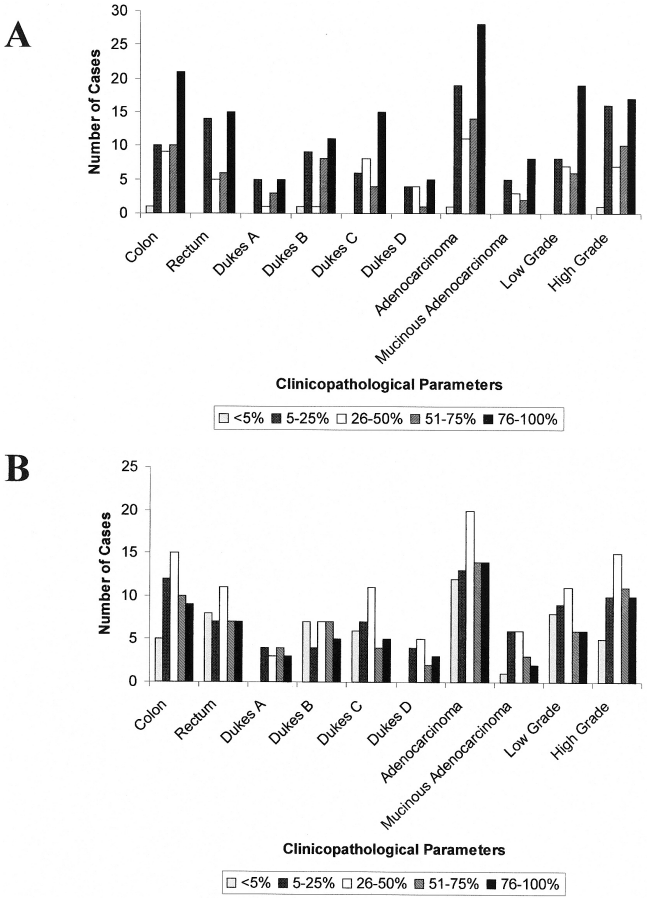

Fig. 3. Graphic representation of the relationship between trypsin **(A)** and TATI **(B)** expression with clinicopathological parameters.

RT-PCR

Trypsinogen 1 and TATI mRNA were detected in positive controls (COLO 205 cells and normal pancreatic tissue) and in all non-malignant tissues (9/9)

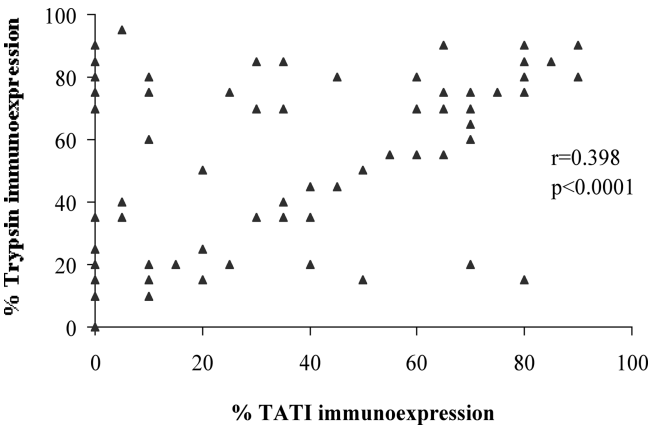

Fig. 4. Correlation between the percentages of cancer cells found positive for trypsin and for TATI in serial sections of colorectal adenocarcinomas.

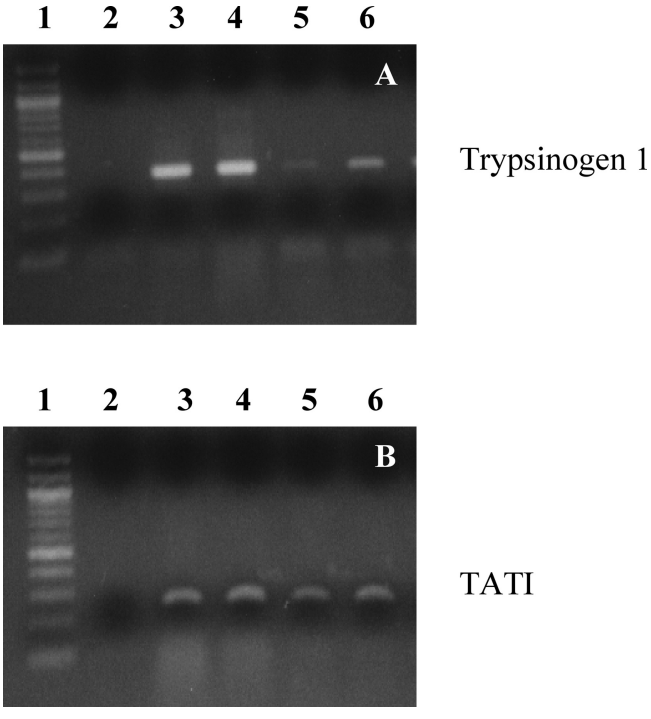

Fig. 5. RT-PCR amplicification with trypsin primers **(A)** and TATI primers **(B)**. Lane 1, molecular weight marker (100 bp ladder). Lane 2, negative control. Lane 3, COLO 205 cells. Lane 4, normal pancreatic tissue. Lane 5, non-malignant mucosa adjacent to colon adenocarcinoma. Lane 6, colon adenocaricnoma Dukes'c.

and adenocarcinomas studied (55/55), as shown in Figure 5.

Discussion

The aim of our study was to assess the parallel expression of trypsin and TATI in colorectal adenocarcinoma specimens, both at the mRNA and the protein level, in comparison with their expression in normal epithelial tissue. We also assessed the possible significance of their expression as a means of differentiation between normal and malignant tissue.

The RT-PCR results of this study concerning TATI expression, agreed with the findings of these past studies. In addition, we also detected trypsinogen 1 in the same tumours that expressed TATI. To our knowledge, there is only one past report of parallel evaluation of trypsinogen 1 and TATI in human neoplasms. Tomita et al (1990) performed Northern blot analysis in 8 colorectal adenocarcinomas and detected a strong signal for trypsinogen 1 in one, and a weak signal in 4 of the cases studied. These tumours also expressed TATI. In our study, TATI mRNA was detected in all colorectal adenocarcinomas examined, in agreement with previous studies (Higashiyama et al., 1990a,b; Tomita et al., 1990). We also detected trypsinogen 1 and TATI mRNA in presumably non-malignant mucosa, obtained at a distance of more than 10cm from the adenocarcinoma.

We have demonstrated distinct staining patterns for trypsin and TATI in presumably normal colonic epithelial cells adjacent to adenocarcinomas. Trypsin was detected in the surface epithelium and TATI was confined to the basal crypts of the mucosa, while the rest of the non-malignant mucosa, adjacent to the adenocarcinoma, remained unstained. These expression patterns were not observed in specimens from normal biopsies, which were negative for both trypsin and TATI. This difference between normal tissue and tissue adjacent to the adenocarcinoma could therefore be an indication that this presumably normal, adjacent tissue could already be pre-malignant.

Our immunohistochemical study has shown trypsin and TATI expression of various degrees in the majority of the cancer cases examined (98.90% and 85.71% respectively). Trypsin may be therefore a far better parameter to discriminate cancer from normal mucosa than TATI, since several tumours not expressing TATI expressed high levels of trypsin, and trypsin expression was absent in only one tumor. To our knowledge, there are four studies of immunohistochemical detection of trypsin or TATI in colorectal cancer. Terada et al. (1997) have shown heterogeneous expression of trypsin in 7 out of 10 adenocarcinomas studied. Another study (Miyata et al., 1999) has shown staining of trypsin in 10 out of 31 cases. Higashiyama et al. (1990a,b) found 80 out of 95 specimens positive for TATI, while Bohe et al. (1990) detected TATI antigen only in normal colonic mucosa, but not in adenocarcinomas (only 2 out of 6 samples gave a weak signal). The differences in the percentage of TATI-immunopositive cases between studies may be due to differences in the processing of the specimens, the sensitivity of the immunohistochemical method used and the specificity of the selected antibodies. Higashiyama et al. (1990a) and Bohe et al. (1990) used TATI-specific polyclonal antisera, while in our study a highly specific anti-TATI monoclonal antibody was applied. Moreover, the negative findings of Bohe et al. (1990) may be attributed to the small number of cases evaluated.

In the present study, we have shown that colorectal adenocarcinomas express trypsin and TATI both at the mRNA and protein level. We have also attempted a semi-quantitative evaluation of the immunohistochemical expression of trypsin and TATI, which resulted in a statistically significant correlation between the degree of expression of the two antigens. This finding of a balance between the protease and its inhibitor suggests the possibility of a physiological role of trypsin and TATI in colorectal adenocarcinomas.

Malignant cells that are able to cross the extracellular matrix, acquire a migratory phenotype. The extracellular matrix is made up of a dense network of laminin, fibronectin and other glycoproteins, collagens, and proteoglycans. The lytic machinery of malignant cells consists of proteases, such as cathepsins, plasminogen activators and matrix metalloproteinases. The activity of proteases is regulated by inhibitors, cytokines, adhesion molecules and growth factors. All these components constitute a net having an active role in metastasis (Verspaget et al., 1998). Trypsin and TATI are thought to be a part of this net (Sorsa et al., 1997; Moilanen et al., 2003).

The significance of trypsinogen in metastasis is still obscure. The role of trypsin is thought to be direct, through its potent proteolytic activity toward a wide variety of extracellular matrix proteins, such as laminin and fibrinonectin, but also an indirect role is possible. Indirect trypsin action is performed through activation of latent forms of metalloproteinases and serine proteases, or may be possible through modulation of the function of various cell-surface proteins, such as growth-factor receptors, integrin and other precursor proteins (Miyata et al., 1999). Expression of trypsin by non-malignant colonic mucosa suggests a role of trypsin in intestinal epithelium homeostasis (Terada et al., 1997). Finally, trypsin as a soluble protein may have effects distant from cancer cells rather than on the cancer cell surface, given the contribution of the stroma in angiogenesis (Egeblad and Werb, 2002).

In this study, we did not detect TATI or trypsin in normal epithelium. Their expression in malignant mucosa suggests a role in the process of invasion of colorectal adenocarcinomas. Nevertheless, trypsin and TATI have been also detected in colorectal adenomas. We speculate that trypsin expressed by adenomas may not contribute to the invasive phenotype, because it may stay inactive. Alternatively, TATI expressed apically by cancer cells may not be able to inhibit trypsin.

The potential biological significance of TATI expression in colorectal cancer may be related to a growth factor-like function that TATI is supposed to have (Freeman et al., 1990b). Although no conclusive evidence has been obtained, TATI has homology with epidermal growth factor and some studies have suggested that it has growth-factor properties. If this is actually the case, a paracrine or autocrine role of TATI though the EGF receptor should be considered.

Elevated serum TATI levels have been reported in patients with various types of cancer (Matsuda et al., 1983). This was also the case in 41.7% of the patients examined in this study (unpublished data). The fact that TATI is produced by colorectal tumours, suggests that neoplasms are a source of TATI in serum and could explain its elevated values.

In conlcusion, we have observed that trypsin and TATI are parallely produced and expressed in colorectal cancer, but we did not detect them in normal mucosa. Our data suggest a role for trypsin and TATI in tumour formation and/or invasion and metastasis in colorectal adenocarcinomas.

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