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BASIC RESEARCH

Expression of tissue factor in pancreatic adenocarcinoma is associated with activation of coagulation

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Abstract

AIM: To study expression of tissue factor (TF) in pancreatic cancer and its role in the development of thromboembolism.

METHODS: TF expression was studied in eight human pancreatic carcinoma cell lines by Northern blot and indirect immunofluorescence. Expression of alternatively spliced TF (asTF) was assessed by RT-PCR. In addition, TF expression was determined by immunofluorescence in pancreatic tissues of 19 patients with pancreatic adenocarcinoma (PCa), 9 patients with chronic pancreatitis (CP) and 20 normal controls. Plasma samples (30 PCa-patients, 13 CP-patients and 20 controls) were investigated for soluble TF levels and coagulation activation markers [thrombin-antithrombin Ⅲ complex (TAT), prothrombin fragment 1 + 2 (F1 + 2)].

RESULTS: All pancreatic carcinoma cell lines expressed TF (8/8) and most of them expressed asTF (6/8). TF expression at the protein level did not correlate with the differentiation of the carcinoma cell line. All but two pancreatic cancer tissue samples stained positive for TF (17/19). In all samples of CP weak staining was restricted to pancreatic duct cells, whereas only a few subendothelial cells were positive in 9/20 of normal controls. TF and TAT levels in PCa patients were significantly elevated compared to controls whereas elevated F1 + 2 levels did not reach statistical significance compared to controls. In CP patients TAT and F1 + 2 levels proved to be significantly elevated compared to controls, although TAT elevation was less pronounced than in PCa patients.

CONCLUSION: We conclude that in addition to the upregulated expression of TF on the cell membrane, soluble TF might contribute to activation of the coagulation system in pancreatic cancer.

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Key words: Coagulation activation; Pancreatic carcinoma; Thromboembolism; Thrombosis; Tissue factor

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INTRODUCTION

Cancer patients are highly susceptible to thromboembolic complications. These thromboembolic complications include venous and arterial thrombosis, migratory thrombophlebitis, pulmonary embolism and disseminated intravascular coagulation (DIC). Idiopathic deep vein thrombosis may be the first clinical manifestation of an occult malignancy and the cancer risk is particularly increased within the first twelve months after the diagnosis of thromboembolism[1].

Patients with idiopathic venous thrombosis have been recognized to have a three to four fold increased likelihood of harboring malignancies[2,3]. Cancer patients have a two to eight fold higher risk of dying after an acute thrombotic event than patients without cancer[4-6]. Furthermore, pancreatic cancer is associated with the highest risk for thromboembolism with an estimated risk ratio of 10[7].

Although the first description of an association of malignant disease with thrombotic events dated back as early as 1865, the underlying mechanisms are poorly understood[8].

Tissue factor (TF) is a key element in the initiation of the extrinsic coagulation cascade. Binding of activated factor Ⅶ to TF results in the activation of factor IX and X ultimately leading to thrombin formation which generates fibrin from fibrinogen and activates platelets[9]. TF is a 49-kD transmembrane glycoprotein belonging to the cytokine receptor family group 2 and contains 263 amino acid residues. Procoagulant activity of TF is tightly regulated at the transcriptional level and the natural inhibitor tissue factor pathway inhibitor (TFPI) can inhibit uncontrolled activation of coagulation. Recently, a variant of TF was identified which results from alternative splicing of the primary RNA transcript. Alternatively spliced TF (asTF) proves to be biologically active[10,11].

Constitutive TF expression is demonstrated predominantly in the brain, lung, placenta, cerebral cortex and kidney[12,13]. Aberrant expression of TF, the principal initiator of blood coagulation, has been postulated to contribute to thrombosis in cancer patients.

Various tumor entities are shown to express TF, including glioma[14], breast cancer[15,16], non-small cell lung cancer[17,18] and pancreatic cancer[19,20].

The present study was designed to systematically investigate the expression of TF by pancreatic carcinoma cells *in vitro* and *in vivo*. Specifically, we focused on the following questions: Do ductal pancreatic adenocarcinoma cells express TF and is the expression intensity related to the degree of tumor differentiation? Is the expression of TF restricted to pancreatic carcinoma cells? Does expression of TF adversely affect blood coagulation in patients as analysed by coagulation activation markers? Is clinical thromboembolism in these patients related to plasma coagulation activation?

MATERIALS AND METHODS

Cell lines

Eight human pancreatic carcinoma cell lines were studied for TF expression: AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-2, PaCa-3, PaCa-44, and PANC-1. The cell lines have been previously studied and characterized[21-23]. The grading of the tumor cell lines as assessed by electron microscopy[21] is listed in Table 1. The expression of asTF was assessed in eight pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-44, PANC-1, NP9, NP29), in two colorectal carcinoma cell lines (DLD-1 and SW48), in the cervical cancer cell line HeLA and in fibroblasts.

Cells were grown in their respective medium (usually RPMI-1640) supplemented with 10% fetal calf serum. For serum deprivation, cells were seeded in tissue culture flasks and incubated with medium containing 10% fetal calf serum for 24 h, then washed twice with sterile PBS, and finally incubated with serum-free medium.

Northern blot

RNA was extracted from exponentially growing cell lines as described earlier[22]. Twenty μg of total RNA was analysed by formamide agarose gel electrophoresis and Northern blotting[22]. Blots were hybridized to a TF cDNA probe[24] and subsequently to a ribosomal cDNA control probe (S138). The 1.4 kb TF-specific insert was labelled with 32P-dATP yielding 5-7 × 10^8 cpm per μg DNA. Filters were hybridized with 1-2 × 10^6 cpm/mL hybridization mix at 42℃ overnight, washed with 2 × SSC at 37℃ for 30 min and 0.5 × SSC at 55℃ for 10 min, dried, and exposed to Kodak XAR film using Kronex lightning enhancer screens. Films were evaluated semiquantitatively according to an established rating. A minimal or no signal representing densitometric measurements < 0.100 was rated 0; a weak signal equivalent to densitometric values 0.100 to 1.0 was rated 1; a signal equivalent to densitometric values 1.0 to 5.0 was rated 2, and a signal equivalent to densitometric readings > 5.0 was rated 3[21,22].

Table 1 Tissue factor expression in eight human pancreatic adenocarcinoma cell lines in relation to the histological differentiation determined by northern-blot analysis (RNA level) and by indirect immunofluorescence (protein level)

	Grade	RNA	Protein
Capan-1	Ⅰ	1[1]	2
Capan-2	Ⅰ - Ⅱ	3	2
AsPC-1	Ⅰ - Ⅱ	3	3
BxPC-3	Ⅱ	3	2-3
PANC-1	Ⅱ - Ⅲ	1	1-2
PaCa-2	Ⅱ - Ⅲ	1	2-3
PaCa-3	Ⅲ	1[1]	3
PaCa-4	Ⅲ	1	1-2

[1] Positive only in the absence of fetal calf serum.

Table 2 Overview of oligonucleotides used for amplification of TF and asTF

Type of primer	Oligonucleotide
Forward	5´-CAGGCACTACAAATACTGTGGCAG-3´
Reverse	5´-TGCAGTAGCTCCAACAGTGCTTCC-3´

TF: Tissue factor; asTF: Alternatively spliced TF.

RT-PCR

For detection of concomitant expression of TF and asTF, cells were washed with PBS. After complete removal of the PBS, 350 μL buffer RLT (RNeasy Mini Kit; Qiagen, Hilden, Germany) plus β-ME (10 μL/mL) was pipetted directly onto the cells. The lysate was processed further according to the manufacturer´s recommendations. Isolated total RNA (1 μg, determined photometrically) was reverse transcribed using Superscript Ⅱ reverse transcriptase (Invitrogen; Karlsruhe, Germany) and oligo-dT primers. The resulting cDNA was amplified in separate tubes using Hot Star Taq (Qiagen; Hilden, Germany). Used primers are listed in Table 2. Oligonucleotides corresponded to TF nucleotides 221-244 and 1013-1036, respectively. TF and asTF were discriminated on a 2% agarose gel as bands of 815 and 656 bp, respectively. The cycling parameters are as follows: initial denaturation and Taq polymerase activation at 94℃ for 15 min, cycling (33 ×) at 94℃ for 45 s, 50℃ for 45 s and 72℃ for 45 s and a final extension at 72℃ for 5 min.

Immunofluorescence

Tumor cell lines grown on glass slides, cryostat sections of pancreatic carcinoma, chronic pancreatitis, and of normal pancreatic tissue were fixed in acetone at -20℃ for 10 min and immunostained by indirect immunofluorescence. The primary mouse monoclonal antibody against TF was clone 5G9[25]. Staining was performed on at least two separate preparations for all tissue sections or cell lines. Staining intensity was graded semiquantitatively as described before[21,22]: a negative staining was rated 0, weakly positive 1, moderately positive 2, and strongly positive 3. This referred to the overall staining intensity. If there were focal positive spots, it was documented separately. Appropriate controls were included throughout this investigation.

Pancreatic tissue

Forty-eight tissue samples were available for further investigation: 19/30 samples from patients with pancreatic carcinoma and 9/13 samples from patients with chronic pancreatitis. Twenty normal tissue samples were obtained from organ donor pancreata[26]. After surgical removal the tissue sample was immediately snap frozen in liquid nitrogen. Five micrometer thick sections were stained with hematoxylin and eosin for histological examination[27].

Patients

The study population comprised 30 patients with pancreatic ductal adenocarcinoma (PCa). Thirteen patients with chronic pancreatitis (CP) and 30 healthy subjects (co) served as controls. Clinically overt thromboembolic events were noted in four patients.

With patients' written informed consent citrate-anticoagulated blood was drawn on the first day of hospitalization prior to the administration of medications potentially interfering with the coagulation system. Platelet-free plasma aliquots were stored at -80℃.

All patients underwent extensive diagnostic work-up including abdominal ultrasound, contrast-enhanced computed tomography and magnetic-resonance tomography, respectively. Suspected thromboembolic manifestations were investigated by doppler ultrasound and when necessary with computed tomography and angiography.

Coagulation studies

Separate plasma aliquots obtained from patients were quickly thawed and utilized for the following commercially available assays according to the instructions provided by the manufacturers. TF antigen concentration was measured using the Imubind® Tissue Factor ELISA kit (American Diagnostics, Greenwich, CT, USA). Thrombin-antithrombin III complex (TAT) was quantitated employing the Enzygnost® TATmicro enzyme immunoassay (Behring, Frankfurt, Germany). Prothrombin fragment 1 + 2 (F1 + 2) was evaluated using the Enzygnost® F1 + 2 kit (Behring, Frankfurt, Germany).

Statistical analysis

Plasma levels of TF, F1 + 2 and TAT are expressed as the means ± SD. Statistical significance was calculated using the two-sided Mann-Whitney test. A P-value of < 0.05 was considered statistically significant.

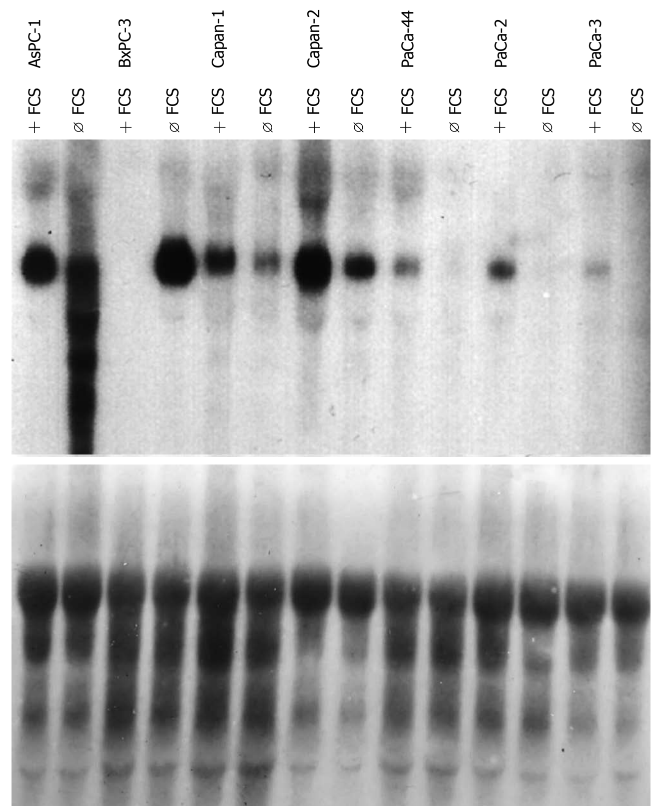

Figure 1 Northern blot of human pancreatic carcinoma cell lines utilizing a cDNA probe for tissue factor (TF) and a ribosomal cDNA control probe (S138). Cell culture conditions with and without (ø) fetal calf serum (FCS).

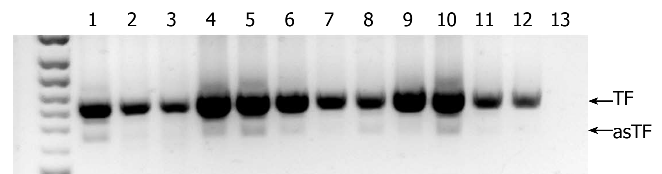

Figure 2 Assessment of TF and asTF expression by RT-PCR. 1: DLD-1; 2: SW48; 3: PANC-1; 4: BxPC-3; 5: PaCa-44; 6: Capan-2; 7: Capan-1; 8: AsPC-1; 9: NP9; 10: NP29; 11: HeLa; 12: Fibroblasts; 13: Negative control.

RESULTS

Expression of TF in pancreatic carcinoma cell lines

All of the eight human pancreatic carcinoma cell lines expressed TF (Table 1, Figures 1, 2, 3A and B). Expression at RNA-level could be modulated in part by serum depletion (Figure 1). All lowly differentiated cell lines (grade Ⅱ and Ⅱ-Ⅲ) exhibited weak TF expression determined by Northern-blot analysis, whereas three of the four highly differentiated cell lines (grade Ⅰ, Ⅰ-Ⅱ and Ⅱ) were found to have a strong TF expression at the RNA level. In contrast, no clear correlation between differentiation and TF protein levels could be demonstrated by immunofluorescence (Table 1).

Expression of asTF in pancreatic cancer cell lines

In addition to TF, the majority of studied pancreatic carcinoma cell lines (6/8) expressed asTF (Figure 2). Furthermore, asTF expression could be demonstrated in the colorectal carcinoma cell lines DLD-1. The colorectal

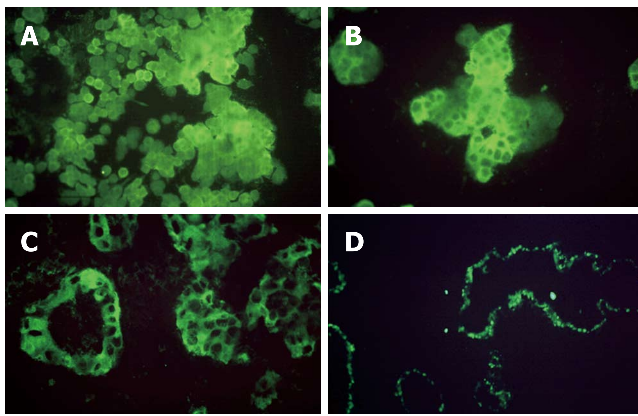

Figure 3 Representative demonstration of tissue factor (TF) expression by immunofluorescence in AsPC-1 (**A**) and CAPAN-1 (**B**) pancreatic cancer cell lines, in tissue of pancreatic cancer (**C**) and chronic pancreatitis (**D**).

Table 3 Immunofluorescence staining analysis of tissue factor expression in patients with pancreatic carcinoma (PCa), chronic pancreatitis (CP) and in healthy controls (Co)

	PCa (*n* = 19)	CP (*n* = 9)	Co (*n* = 20)
Total immunoreactivity	17 (84%)[1]	9/9 (100%)[2]	9/20 (45%)[3]
Grade 3	4	0	0
Grade 2	7	0	0
Grade 1	6	9[+]	0
Negative	2	0	11

[1]*P* = 0.027 *vs* controls; [2] A few positively stained epithelial duct cells; [3] Expression restricted to subendothelial cells.

carcinoma cell line SW48, the cervical cancer cell line HeLA and fibroblasts did not express asTF.

Expression of TF in pancreatic tissue

Of the 19 pancreatic carcinoma tissue samples nearly all (17/19) were positive for TF (4/19: grade 3, 7/19: grade 2, 6/19: grade 1) and only two were negative (Table 3, Figure 3C). The amount of TF expression in tumor tissue did not correlate with the grading of the tumor (Table 3).

All tissue samples obtained from chronic pancreatitis demonstrated positive staining of epithelial pancreatic duct cells (Table 3, Figure 3D). However, staining intensity was rated weakly in these cases. Nearly half of all normal control tissue samples exhibited only weak staining of a few subendothelial cells (9/20) (Table 3).

Plasma concentrations of TF in patients with pancreatic cancer and healthy controls

TF plasma concentrations in 30 patients with pancreatic cancer were significantly enhanced versus 30 healthy controls (288 ± 41.4 ng/L *vs* 139 ± 9.7 ng/L, *P* = 0.005) (Figure 4).

Coagulation studies

Plasma levels of TAT in patients with pancreatic carcinoma (PCa) were more than twenty-times higher than in normal controls (89.4 ± 38.8 μg/L *vs* 4.0 ± 3.5 μg/L, *P* = 0.002). Interestingly, they were also significantly enhanced

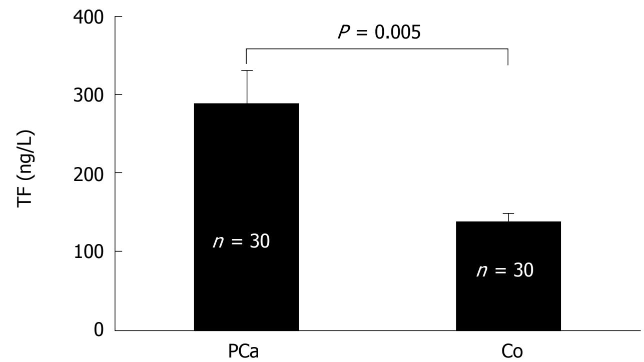

Figure 4 Plasma concentrations of tissue factor (TF) in patients with pancreatic cancer (PCa) versus controls (Co). Bars indicate means and standard deviation.

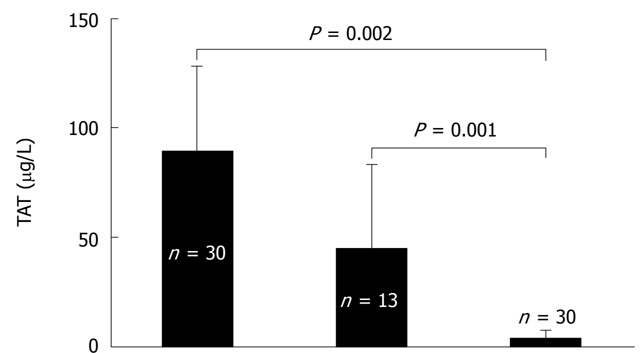

Figure 5 Plasma concentrations of thrombin-antithrombin complex (TAT) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

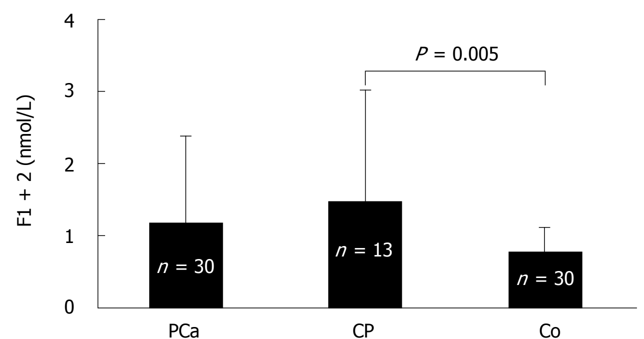

Figure 6 Plasma concentrations of prothrombin fragment 1 + 2 (F1 + 2) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

in patients with chronic pancreatitis (CP) versus healthy controls (45.1 ± 37.9 μg/L *vs* 4.0 ± 3.5 μg/L, *P* = 0.001) (Figure 5). However, higher levels in PCa patients compared to CP patients did not reach statistical significance.

Plasma levels of prothrombin F1 + 2 proved to be higher in patients with pancreatic adenocarcinoma although not reaching statistical significance (1.16 ± 1.56 nmol/L *vs* 0.75 ± 0.34 nmol/L, *P* = 0.16), whereas significant elevation of F1 + 2 was observed in patients with chronic pancreatitis versus controls (1.46 ± 1.23 nmol/L *vs* 0.75 ± 0.34 nmol/L, *P* = 0.005) (Figure 6). There was no correlation between the plasma levels of

Table 4 Patients with pancreatic carcinoma complicated by clinically overt thromboembolism

Case	Sex/age	Stage[1]	Grade	TF[2]	TF (pg/mL)	Location of thromboembolism
1	m/65	4	II	3	291	Splenic vein thrombosis
2	m/69	4	II	2	nd	Mesenteric artery thrombosis
3	f/61	4	II	1[3]	391	Upper jugular vein thrombosis
4	m/62	4	II	2	nd	Pulmonary embolism

[1] Staging according to UICC; [2] Results from immunohistochemistry, i.e. immunofluorescence for TF; [3] TF was focally positive; nd: not determined.

TAT or F1 + 2 and the extent of TF expression in the pancreatic tumor tissue (data not shown). However, there was a strong correlation between TAT and F1 + 2 plasma levels ($P < 0.05$).

Patients with pancreatic cancer and thromboembolic complications

Four of the TF-positive patients had thrombosis, as revealed by doppler ultrasound, computed tomography and/or angiography (Table 4). All patients with thrombosis had a stage 4 pancreatic carcinoma. In two cases TF plasma levels were available which were increased by a factor of 2 and 2.8, respectively, compared to the mean value of TF plasma levels in healthy controls.

DISCUSSION

Our study proved expression of TF by epithelial tumor cells in pancreatic adenocarcinoma *in vitro* and *in vivo*. In patients with pancreatic cancer, a hypercoagulative state could be confirmed by increased concentrations of the TAT in conjunction with elevated TF plasma levels. Four patients expressing TF in their tumors developed clinically overt thromboembolism.

The presence of a malignant disease dramatically increases the risk for thromboembolic events. Immobility, indwelling catheters, surgical procedures and chemotherapy represent risk factors rendering cancer patients for thromboembolism[28]. In addition, direct activation of the coagulation cascade by cancer cells is considered a key feature of the well-established increased risk for thrombotic events. TF plays a central role in activating coagulation and enhanced expression is implicated in diseases with enhanced thrombotic features like cancer[29], atherosclerosis[30], sickle cell disease[31] and sepsis[32].

We analyzed the expression of TF in eight well characterized cell lines of ductular adenocarcinoma[23]. Without exception all showed TF expression at RNA and protein levels. Highest RNA levels were found in the well-differentiated cancer cell lines (Capan-2, AsPC-1, BxPC-3). However, in relation to differentiation no clear correlation of TF expression could be observed at the protein level. The fact that the amount of TF protein levels did not strictly correspond to RNA levels suggests that posttranscriptional regulation might be of importance in TF expression. We could not confirm the finding of Kakkar that expression of TF was most prominent in poorly differentiated tumors[20].

In addition to expression of TF, the majority of studied pancreatic carcinoma cell lines expressed the recently characterized asTF[10,11]. This splice variant contains the extracellular domain of TF, but lacks the transmembrane and cytoplasmatic domain. Exon 4 is spliced directly to exon 6 leading to a frameshift so that the translated peptide comprises residues 1-166 of the extracellular domain of TF and a unique C terminus (residues 167-206). AsTF proved to have full pro-coagulant activity. The expression of asTF might contribute to systemic hypercoagulopathy in pancreatic cancer.

Corresponding to the TF expression in pancreatic carcinoma cell lines we demonstrated, in the majority of tissue specimens of patients with pancreatic cancer, TF expression in tumor cells, which showed a high variability in the expression rate determined by immunofluorescence. Recently, upregulated TF expression in colorectal cancer cells was ascribed to *K-ras* mutations[33]. As mutations in the *K-ras* oncogene are an early event in the development of pancreatic cancer and as these mutations are found in the majority of pancreatic cancer patients, mutational activation of *K-ras* might have a contributory role in upregulated TF expression[34]. Further studies are warranted to clarify whether inactivation of the tumor suppressor gene PTEN and hypoxia are involved in enhanced TF expression in pancreatic cancer as it was recently demonstrated in glioblastoma[35]. Lately, Nitori and co-workers determined TF expression in pancreatic cancer by immunohistochemistry and found an association of high TF expression with metastasis and a low survival rate[36].

To our knowledge this is the first report demonstrating an enhanced TF expression in tissues of patients with chronic pancreatitis which was confined to pancreatic duct cells. This was a surprising result as tissue of chronic pancreatitis patients was initially intended to be included in the study to serve as a negative control. Previous data have shown that the exposure of endotoxin, tumor necrosis factor (TNF)-α and interleukin (IL)-1 results in upregulation of TF expression *in vitro*[37-40]. Proinflammatory cytokines secreted by the inflammatory infiltrate in chronic pancreatitis might be an explanation for the upregulated TF expression[41]. In contrast, only weak staining of a few subendothelial cells was found in control tissue specimens. Upregulated TF expression in the tissue of chronic pancreatitis patients might have a contributory role in the development of thrombotic events, which is a well-known complication in chronic pancreatitis.

TF plasma concentrations in patients with pancreatic cancer were found to be more than twice as high than in normal controls which might stem from asTF or from TF-containing microparticles and from TF which is secreted by activated leukocytes, respectively[42-44]. Further studies are needed to prove that the source of TF in the plasma of patients with pancreatic cancer is the tumor itself.

In parallel to enhanced TF expression, markers of thrombin formation (TAT, F1 + 2) were elevated in the plasma of patients with pancreatic cancer and chronic pancreatitis. A procoagulatory state in patients with chronic pancreatitis might facilitate the generation of portal and splenic vein thrombosis, which is commonly seen in this patients' group.

Four patients with pancreatic cancer had concurrent venous thrombosis or pulmonary embolism. TF expression was highly variable but all patients had a stage 4 carcinoma. Apparently the tumor load and tumor progression is a more powerful prognostic marker for thrombotic complications than TF expression in pancreatic cancer. This is in accordance with data showing that the stage of colorectal cancer most consistently correlates with the risk for thromboembolism[45].

In this study we focused on TF expression, taking into account that undoubtedly multiple factors modulate hypercoagulative state.

Tissue factor pathway inhibitor (TFPI) is an important factor limiting the effects of TF. In a recent study low levels of TFPI were associated with an increased risk of venous thrombosis[46]. Mice deficient of TFPI exhibited an increased rate of thrombosis and accelerated atherosclerosis[47]. Both pancreatic carcinoma and chronic pancreatitis produce a rich stroma[48]. We demonstrated that epithelial tumor cells can synthesize and deposit extracellular matrix proteins in pancreatic carcinoma[21,49]. Fibronectin itself which is expressed by pancreatic tumor cells[21] is capable of activating the coagulation cascade[50].

In addition to its haemostatic potential, the TF expression by tumor cells has implications in tumor biology[51,52]. Belting and coworkers have shown that TF directly promotes angiogenesis through protease-activated receptor-2 (PAR-2) signaling[53]. Upregulation of the urokinase receptor in pancreatic cancer by TF enhances tumor invasion and metastasis[54]. Pancreatic carcinoma cells possess a functioning thrombin receptor, and stimulation with thrombin representing the product of the coagulation cascade causes cell proliferation[55]. Furthermore, thrombin has a proangiogenic function[56,57].

In summary, enhanced TF expression in pancreatic adenocarcinoma in conjunction with elevated TF plasma levels might have a crucial role in hypercoagulopathy, resulting in thromboembolism.

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