Risk factors of thrombosis in cancer : the role of microparticles

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8 Microparticle-associated tissue factor activity, venous thrombosis and poor survival in pancreatic cancer patients

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Summary

Background

Patients with pancreatic adenocarcinomas have a high incidence of venous thrombosis (VT). Histological findings in pancreatic carcinoma indicates that high grade tumours are associated with expression of tissue factor (TF) in tumour cells, suggesting that TF could indeed be involved in processes such as clotting, angiogenesis and metastasis, which determine the clinical outcome. Microparticles (MP), released from tumour cells expressing TF, might be a mediator of thrombosis in patients with pancreatic carcinoma.

Methods

62 patients with ductal adenocarcinoma of the pancreas, 22 patients with locoregional disease and 40 with distant metastatic disease were studied. VT was confirmed by ultrasound or CT-scan. MP were isolated from blood and MP-associated TF activity determined. In 27 patients, immunohistochemistry was performed to assess expression of TF in tumour tissue.

Results

Thrombotic events developed in 12 of the 62 patients (7 cases with deep vein thrombosis, 3 cases with pulmonary embolism and 2 cases with both). There was no difference between the total number of MP, platelet-derived MP and platelets in pancreatic cancer patients with or without thrombosis. All patients with VT had elevated MP-TF activity with a median of 1891 fM Xa/min (range 510-12,344), whereas only 6 (12%) patients without VT had elevated MP-TF activity (median 463 fM Xa/min; range 334-855). There was no clear association between TF expression on the tumour and MP-TF activity or development of VT. Median survival following diagnosis of ductal pancreatic adenocarcinoma was 4 months (range 1-20) and 6 month-mortality was 30 % (*n* = 17). MP-TF activity and clinically manifest venous thrombosis were the only factors associated with poor survival.

Conclusions

Microparticles bearing active TF may play an important role in the pathogenesis of cancer-related thrombosis and may serve as prognostic marker for thrombosis and survival in patients with adenocarcinoma of the pancreas.

Introduction

Venous thromboembolism (VT) is common in patients with malignant disease (1-3) and numerous studies documented the association between cancer and VT. Although abnormalities in various proteins involved in the clotting cascade have been investigated, only known risk factors such as FV Leiden or prothombin gene mutation has been shown to increase the risk of VT two-fold (4), whereas elevated levels of FVIII has not been shown to play a major role in cancer-related VT.

The cause of the excessive risk in certain cancers has not been elucidated, but differences with regard to the excess risk for thrombosis exist between the various types of malignancies (4). Carcinoma of the pancreas, a relatively rare tumour of which in the last decades the incidence is increasing, seems to have an inherent and unique ability to induce a hypercoagulable state that leads to clinically significant thrombosis (5). Ogren *et al*. (6) reported a large series of 23,796 standardised autopsies performed between 1970 and 1982 and representing 84% of all in-hospital deaths in an urban Swedish population.The overall PE prevalence was 23%, and 10% of the population had a fatal PE. Forty-two per cent of pancreatic cancer patients had PE (OR 2.55; 95% CI 2.10-3.09). Adenocarcinoma and metastatic cancer were independently associated with PE risk (OR 1.27; 95% CI 1.16-1.4 and OR 1.10; 95% CI 1.01-1.20 respectively). They concluded that the risk of PE in cancer patients largely depends on cancer site and spread of the disease, but also on histological type and in particular reported an intriguing excess independent risk for pancreatic cancer warranting further research.

Recently, Tissue Factor (TF), initiator of coagulation, has been suggested to play a central role in the association between cancer and thrombosis. Studies indicated that perhaps TF as well as membrane-bound TF, *i.e*. microparticle **(**MP**)**-associated TF (7), may be one of the mechanism by which tumour growth and clotting form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. Expression of TF is associated with poor differentiation of tumours and poor prognosis (8;9) but also cancer patients who develop thrombosis have a poor survival as compared to those that do not (10;11). Patients with adenocarcinoma of breast or pancreas were found to have significantly increased levels of MP-associated TF activity in their circulation and MP-associated TF activity correlated with VT and the presence of circulating MP expressing the epithelial antigen MUC1 -most likely derived from malignant cells (7). Patients with high level of TF-activity on MP that also expressed mucin had a significantly lower survival

rate than those with low TF-activity and no mucin expression. Thus, TF and MUC1 on MP may be factors which play a decisive role in the pathogenesis of the prothrombotic state in disseminated mucinous adenocarcinoma patients.

Patients with mucinous adenocarcinomas of the pancreas are thus at high risk of venous thromboemblism, have a poor prognosis and short survival once the diagnosis is made, whereas effective systemic treatment options are of very limited value. For this reason, this patient population provides an unique opportunity to study the biological role of tumour-related compounds without intervention of other factors which may affect the occurrence of thrombosis, such as chemotherapy.

Interestingly, the endocrine, but not the exocrine, cells of the pancreas were found to synthesise and secrete active TF (12). Aadenocarcinomas arising from ductal exocrine, which often produce large amounts of mucins, are the most common malignancy in the pancreas, and in particular high grade pancreatic cancer have been reported to express tissue factor (13;14). The extracellular domain of TF is involved in initiating the clotting cascade, whereas the intracellular domain has been reported to be involved in angiogenesis and the process of cell invasion (15). Therefore, importantly, TF expressed in pancreatic ductal carcinoma cells may be involved in tumour progression, which may be related to the clotting activity of TF, but also to TF-mediated activation of angiogenesis and tumour cell invasion. Production and release of tissue factor may thus provide an explanation for the aggressive biological behaviour of pancreatic carcinoma.

We therefore decided to study the expression of TF in invasive pancreatic cancer, circulating MP bearing active TF and thromboembolic events in relation to survival time in pancreatic cancer patients.

Materials and methods

Patients

Sixty-two consecutive patients diagnosed between 2003 and 2007 with pancreatic adenocrcinoma were identified and evaluated to document the incidence of VT and the predisposing factors. Of these patients, 13 underwent pancreatic resections for pancreas ductal adenocarcinoma and plasma samples were collected pre-operatively with the tumour still in situ. Another 9 pancreatic cancer patients were diagnosed with locally advanced irresectable tumours, while 40 patients were diagnosed as metastatic disease.

In one patient we collected two samples, one sample before surgery and one sample at the time of metastatic disease. Staging of the diseases occurred by CT scan and in some cases also during exploratory surgery. Plasma samples were always collected before the start of any systemic treatment or before surgery. Tumour type was pathologically confirmed in all patients. All individuals signed institutional review board approved consent.

MP isolation

Blood was collected in 1/10 volume of 3.2% trisodium citrate (Becton Dickinson, San Jose, California) and prepared immediately by high-speed centrifugation as described previously (7). Platelet-poor plasma (source of MP) was carefully removed, snap frozen in liquid nitrogen and stored at −80ºC for future analysis. MP-containing plasma was, washed, and resuspended in 1/10 of the original volume buffer (154 mM NaCl, 1.4 mM sodium phosphate, 10.9 mM trisodium citrate, pH 7.4). For the measurement of MP-associated TF activity, MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate. Assays were performed on MP isolated from deep frozen plasma, allowing comparison of MP from different individuals in the same exercise as previously described.

Flow cytometric analysis of MP

Flowcytometric analysis of MP was performed using Allophycocyanin (APC)-labeled AnnexinV from BD Biosciences/Pharmingen (San Jose, CA) and cell-specific monoclonal antibody (mAb) or isotype-matched control antibody labeled with phycoerythrin (PE): anti-CD41-PE (clone P2, IgG_1), mouse IgG_1-PE (X40) from BD Biosciences/Pharmingen (San Jose, CA). MP were double-stained with Annexin V and anti-CD41-PE, analyzed for 1 minute with Cell Quest software (Becton Dickinson, San Jose, CA), and identified by their characteristic forward and side scatter, and by their ability to bind Annexin V and cell-specific moAb. The number of MP/L plasma was calculated as previously described.

Tissue factor activity assay

The TF activity in MP preparations was measured in 96 wells plates at room temperature by determining the FVII-dependent factor Xa (FXa) generation as previously described (7). Results are reported as the MP-associated TF-activity/ ml plasma (fM Xa/min). For all samples the FVII dependent FXa generation was found to be sensitive to anti-tissue factor antibodies. The median and mean TF activity in the MP isolated from the plasma

of healthy subjects (MP-associated TF activity) did not differ (129 versus 132 fM Xa/min) and TF activities higher than the mean + 3 SD (> 273 fM Xa/min) were considered to be elevated.

Circulating mucins

Circulating mucin Ca 19.9 (normal values ≤ 37 kU /L) were determined in plasma utilizing commercially available immunoenzymatic assays on an IMx (Abbott Diagnostica, IL, USA). The assay was routinely performed to measure different isoforms of these mucins.

Immunohistochemistry

Tissue sections of formalin-fixed, paraffin-embedded tumour biopsies or resection specimen of pancreatic tumour tissue were deparaffinised, after an overnight drying step, with xylene and rehydrated in increasing percentages of ethanol. After an antigen retrieval step, sections were incubated with the following mAbs or isotype-matched control antibodies: anti-mouse IgG_1 from BD Biosciences/Pharmingen (San Jose, CA), mouse-anti-human TF (4509) from American Diagnostica, (Stamford, CT) or mouse-anti-human epithelial membrane antigen (clone E29) from DakoCytomation (Glostrup, Denmark). Antibodies were diluted in Dako Real Antibody diluent to decrease background staining and avoid the need for additional blocking steps. After incubation, sections were washed in PBS and endogenous peroxidase was blocked in Peroxidase block solution (DakoCytomation, Glostrup Denmark) for 15 min. Thereafter, EnVision immunoenzymatic system was used. All sections were reviewed by two independent persons who were blinded with respect to the results of the MP-TF activity assays. TF and mucin expression was scored based on percentage of positively staining tumour cells as well as intensity of the staining.

Statistical Analysis

We compared means and proportions and constructed confidence intervals based on normal and binomial distributions. Kaplan-Meier curves were constructed to assess survival and cox models were used to calculate hazard ratios. All analyses were performed using SPSS for Windows, version 14.0 (SPSS Inc, Chicago, IL).

Results

Table 1 shows the patients characteristics of the 62 pancreatic cancer patients with and without VT. Twenty-one (33%) patients received chemotherapy after collection of plasma samples. In 13 (21%) pancreatic cancer patients, VT (*i.e.* in 8 patients deep vein thrombosis, in 3 pulmonary embolism, and in 2 both) was observed. Median survival following in the whole group of ductal pancreatic adenocarcinoma patients was 4 months (range 1-20 months) and mortality at 6 months was 31% (*n* = 19). Survival was not associated with age, sex, or chemotherapy.

Table 1. Characteristics of pancreatic carcinoma patients who did or did not develop VT.

	Patients with VT		**Patients without VT**	
	***n* = 12**	**(%)**	***n* = 50**	**(%)**
Surgical resection	1	(8.3)	12	(24.0)
Distant metastases	11	(91.7)	30	(60.0)
Alive at 3 months	2	(16.7)	32	(64.0)
Alive at 6 months	1	(8.3)	18	(36.0)
Overall survival				
median (mo)	1		5	
range (mo)	1-11		1-20+	
CA 19.9 (UK/L)				
median	37,751		593	
range	5-197,000		1-56,365	
Platelets (x 10^9/L)				
median	323	(100)	238	(100)
range	168-678		120-581	
MP total (x 10^6/L)				
median	4,933	(100)	5,890	(100)
range	1,849-23,386		1,500-13,897	
P-MP (x 10^6/L)				
median	4,281	(100)	5,370	(100)
range	1,762-17,859		1,000-12,673	
MP-TF activity (fM Xa/min)				
median	1,891	(100)	134	(100)
range	102-12,344		120-581	

There was no difference between the total number of circulating MP and platelet-derived MP as well as absolute platelet count. Total number of circulating MP was not associated

with MP-TF associated activity, but there was a strong correlation between MP-TF activity and VT $P < 0.000$. Furthermore in all patients with VT, mucin-bearing MP were found whereas this was found in 28 % of the pancreatic cancer patients without VT. More pancreatic cancer patients with VT had metastatic disease as compared to those without VT (OR 7.97 CI 95% 0.95-66.5)

Patients with distant metastases in other organs and/or lymph nodes had a much shorter survival as compared to patients in whom no metastases were detected (estimated median survival 4.0 CI 95% 2.4-5.6 versus 12.0 CI 95% 9.67-14.32; $P < 0.001$); HR for death 3.0. Furthermore, survival was significantly reduced in patients with both elevated levels of circulating MP-associated TF activity and a thrombotic event, MP-associated TF activity and VT were independently associated with a short survival. Furthermore, mucin-producing potential as evidenced by expression of mucin in the primary tumour and/or secretion of CA19.9 mucin in the circulation, were found to be significant predisposing factors with respect to occurrence of VT or survival.

In the 27 available tumour specimens, in mucin was expressed by the tumour cells in almost all cases, but TF in slightly less than fifty percent of the tumours. In most cases a low number of tumour cells were found to express TF. In the 7 patients with elevated MP-TF activity of whom tumour specimens were available, in 55% tissue factor was expressed by tumour cells. Thus no clear correlation between MP-TF activity in a later stage of the disease and tumour TF expression in the original biopsies could be found in this small series. Only one tumour was poorly differentiated, precluding assessment of a correlation between tumour grade and TF expression. In 6 (50%) of the 12 patients who presented with VT and who all had elevated MP-TF activity, tumour specimens were available for TF expression; 2 (33%) of these six patients had TF expression in their tumour. In 3 patients with elevated MP-TF activity but no thrombosis, tumour specimens were available, and in 1 of the 3 tumour samples TF expression was observed in the tumour ducts.

Discussion

We found a high incidence of VT in patients with pancreatic adenocarcinoma and those patients who developed VT presented with markedly elevated levels of circulating MP-TF activity. The majority of patients with thrombosis had metastatic desease and only the malignancy itself as risk factors of thrombosis. Furthermore, MP-TF activity and occurrence of VT were associated with poor survival.

In contrast to Khorana *et al*. (13) and Nitori *et al*. (14) we found TF expression in a low percentage of pancreatic tumours. This discrepancy may relate to the limited number of tumour specimens available and sampling error due to low numbers of malignant cells present in most of the specimens. Mostly, tumour biopsies were rather small, and marked tumour cell heterogeneity present, accounting for a failure to find TF expressing cells if present. Similar to what has previously been described by Moberg *et al*. (12), we always found marked TF expression in the pancreatic islets, precluding that our staining method failed to detect TF. Perhaps, differences in semi quantitative scoring system applied by Khorana *et al*. (13) and Nitori *et al*. (14) explains the lower expression rate of TF in pancreatic carcinoma in our data. Independent of the TF expression by tumour cells in the original specimens, still selection of predominantly TF-expressing tumour cells may have occurred and those cells could have an advantage in the process of metastasis. When released into the circulation, such cells would contribute further to formation of microparticles and perhaps also to the observed thrombotic events and poor survival of the patients.

The role of tissue factor and its inhibitor in thrombosis is further substantiated by other findings, in which elevated circulating TF and tissue factor pathway inhibitor was found in patients with other diseases (16,17) such as cardiovascular diseases and diabetes mellitus. Elevated numbers of TF-expressing microparticles correlated with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Involvement of TF in the pathogenesis of increased risk to develop thrombosis also comes from observations made in women without cancer. Interestingly, Smith *et al*. (18) recently observed that out of 24 coagulation, anticoagulation, fibrinolysis and antifibrinolysis candidate genes, only the tissue factor pathway inhibitor gene was globally associated with the risk of VT in postmenopausal women. Although the findings of Smith *et al*. (18) cannot be generalized to men or younger women, their findings underscore that TF and its inhibitor protein may be important factors also in the well-recognized increased risk of VT in patients with cancer.

Future therapeutic intervention of in the clotting cascade that may be initiated in cancer patients by MP which carry active TF, possibly arising from pancreatic carcinoma cells themselves, could lead to abrogation of the clotting cascade and perhaps improvement of survival. One way could be by blocking the active site of TF with specific antibodies or by administration of candidate drugs for inhibition of TF activity *in vivo*, such as reactive site-inactivated factor VIIa. Patient selection should be done based on

the MP-TF activity assay of plasma. Furthermore, further investigation of polymorphism of genes involved in the clotting cascade is relevant to determine whether this modulates the risk to develop serious thromboembolic complications and poor survival.

In conclusion, this study indicates that unique intrinsic tumour procoagulant characteristics and perhaps tumour-derived microparticles bearing active tissue factor are present in pancreatic adenocarcinoma patients who suffer from thromboembolic complications. Measurement of circulating MP-TF activity identifies patients at high risk for VT and poor survival. Although we could not reproduce the TF expression on pancreatic cancer, TF represents an attractive therapeutic target in pancreatic cancer patients to serve as anti-tumour agent and anti-thrombotic agent and to approve survival in those patients.

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