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Identification of a thrombin receptor with factor Xa receptor and tissue factor in human pancreatic carcinoma cells

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Abstract

Venous thromboembolism is a common feature of pancreatic cancer. The underlying mechanism is unclear, but is likely to involve thrombin generation on the cell surface. Human pancreatic carcinoma cell lines (n=8) have been studied immunohistochemically for the expression of tissue factor, factor Xa receptor, and thrombin receptor. Each antigen had a distinct pattern of immunoreactivity in cell membrane and cytoplasm. Tissue factor was predominantly localised to the membrane, whereas thrombin and factor Xa receptor were largely cytoplasmic in distribution. The results support the hypothesis of a coagulation cascade that starts with tissue factor, leads to thrombin generation, and might confer a biological advantage on tumour cells.

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Keywords: Thrombin receptor, pancreatic cancer.

Thrombosis is a common complication of malignant disease and pulmonary embolism is the second commonest cause of death in cancer patients.¹ Adenocarcinoma of the pancreas is associated with a particularly high incidence of thromboembolism. We have previously reported that the expression of the cell surface procoagulant tissue factor (TF), the physiological initiator of blood coagulation, correlates with histological grade in this disease; normal pancreatic tissue shows no immunoreactivity whereas poorly differentiated tumours have strong immunoreactivity in nearly 80% of

cases.² This work supported our observations that the procoagulant activity in human pancreatic carcinoma cell lines is very high, varying from 14 to 6600 thromboplastin units, with at least 86% of this activity being TF dependent in each case.³ These cells also possess a high prothrombinase activity, a prerequisite to efficient cell surface thrombin generation.

Thrombin is generated by tumour cells in large quantities, therefore explaining the histological observation of peritumoral fibrin deposition⁴ and providing a possible mechanism for the hypercoagulable state seen in cancer patients. However, thrombin possesses a number of important functions other than its role in haemostasis, including effects on endothelial cells such as promotion of arachidonic acid metabolism and release of tissue factor, von Willebrand factor, tissue plasminogen activator, and plasminogen activator inhibitor.⁵ In tumour cells it has been shown to promote synthesis and release of urokinase plasminogen activator.⁶ It is also an important mitogen to normal cells such as fibroblasts and smooth muscle cells that play a key role in atherogenesis.⁷ Thrombin is also mitogenic to tumour cells, potentiating the proliferative response to insulin, epidermal growth factor, and transferrin.⁸

Other activated serine proteases generated as a result of the activation of coagulation have roles outside normal haemostasis and are mitogenic. Of particular interest is factor Xa, the factor occupying the pivotal position in the normal coagulation pathway. Recently a specific receptor (effector cell protease receptor-1 (EPR-1)) for this serine protease has been identified and cloned and shown to be of functional significance.⁹ In this study we have

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used immunohistochemical techniques to determine whether TF is colocalised with EPR-1 and human thrombin receptor on the pancreatic tumour cell surface.

Methods

CELL CULTURE

Eight human pancreatic carcinoma cell lines were investigated. Cell lines were cultured in RPMI 1640 (Gibco) plus 10% fetal calf serum (FCS) (Gibco), in 95% O₂ and 5% CO₂. Tumour cell lines studied were CaPan2, BXPC3, Mia Paca, Panc 1 (ATCC), and 818·1, 818·4, 818·7, PT45 (Dr H Kalthoff, University of Kiel). Cells were studied on two separate occasions after four passages, and on each occasion in duplicate. Cell lines were regularly screened for mycoplasma contamination using a kit (Gene-Probe), and the growth medium was supplemented with penicillin and streptomycin to prevent bacterial or fungal contamination.

Immunohistochemical studies

Cells (1×10^4) were grown on glass microscope slides with detachable plastic wells (Costar) until subconfluent under standard conditions. At this point, when the cells formed a monolayer on the glass slide the well was detached and the cells were washed thoroughly with phosphate buffered saline (PBS) and fixed with methanol 1% (BDH). The following antibodies were used to detect the antigens: (a) tissue factor: α 243 TF polyclonal antibody raised in the rabbit against TF220 the recombinant soluble form of human TF, and affinity purified (Dr E G D Tuddenham, Clinical Sciences Centre, RPMS, London)²; (b) human thrombin receptor: anti-HTR polyclonal antibody raised in the rabbit against the human thrombin receptor¹⁰; (c) effector cell protease receptor - 1 (EPR-1) (also known as the factor Xa receptor)⁹: (i) 9B4 monoclonal antibody raised against the EPR-1 extracellular form of the receptor, and (ii) anti-EPR-2 monoclonal antibody raised against the cytoplasmic form of the receptor.

In each case, 50 μ l of antibody were placed on each slide and incubated for 1 h at room temperature. Slides were rinsed three times in Tris buffered saline (50 nmol Tris HCL at pH 7.6). The second antibody was a 1:300 dilution of biotinylated saline anti-rabbit or anti-mouse IgG (1 g/l Dako), which was further incubated for 1 h at room temperature.

Slides were rinsed, and the third layer of Avidin-Biotin Complex (Dako) was added. After incubation for 45 min and rinsing, the colour reaction was developed using diaminobenzidine hydrochloride (DAB) with 0·037% hydrogen peroxide for 10 min. Negative controls comprised omission of the primary antibody, and substitution with non-immune serum and an irrelevant antibody. There was no evidence of non-specific immunoreactivity with any of these controls.

The immunoreactivity for each antigen was assessed semiquantitatively (0=no positive

Immunostaining for tissue factor (TF), factor Xa receptor (EPR-1, EPR-2), and thrombin receptor (anti-HTR)

	TF	EPR-1	EPR-2	Anti-HTR
PT45	+++	+/-	-	+/-
CaPan2	+++	++	+	++
BXPC3	+++	++/+	+++	++/+
818·7	++	+/-	+	+/-
818·1	++	++	++	+
818·4	++	++	++	++
Mia Paca	+++	+++	++	+++
Panc 1	+	++	++/+++	++

staining to +++ = intense membrane immunostaining). The homogeneity of staining was determined together with the localisation of staining and its reproducibility. Each slide was assessed by two independent observers.

Results (table)

Each of the antigens studied—tissue factor, thrombin receptor and factor Xa receptor (EPR-1, EPR-2)—had a distinct pattern of immunoreactivity. For tissue factor, there was definite membrane immunostaining in every cell line (the strongest being in lines PT45, CaPan2, BXPC3, and Mia Paca), as well as weaker cytoplasmic localisation of antigen. In the case of the human thrombin receptor, there was a cytoplasmic membrane immunoreactivity in all cases, but also a faint membrane localisation of antibody in a small proportion of cells. There was a wide range of immunostaining between cell lines, Mia Paca having the strongest reactivity. In the case of the factor Xa receptor, antibodies against both isoforms of the receptor EPR-1 and EPR-2 gave more or less identical staining patterns with cytoplasmic localisation, often with a perinuclear concentration.

Discussion

The study shows that tissue factor, factor Xa receptor, and thrombin receptor are expressed by human pancreatic carcinoma cells. Although tissue factor has been recognised to be an important procoagulant in a number of tumour cell lines,¹¹ neither factor Xa receptor nor thrombin receptor have previously been described in any malignant cell types. Expression of cell surface tissue factor leading to the generation of factor VIIa is responsible for the activation of the extrinsic pathway of coagulation,¹² and this in turn results in the factor VIIa dependent activation of factor X. Previous investigations from our laboratory³ have shown that pancreatic tumour cells in culture possess the ability to form the cell surface prothrombinase complex. This complex of activated factors X, V, calcium ions, and cell surface phospholipid is responsible for the conversion of prothrombin to thrombin. The prothrombinase complex represents an efficient amplification mechanism for the activation of thrombin and, in the case of the cell surface, a clear method for localisation of the generation of this serine protease. Although the relevance of a specific receptor for factor Xa has yet to be elucidated, its role in leucocyte biology (the

cell type where it was originally described and from which it was cloned⁹) suggests that factor Xa receptor may play a role in both localising and enhancing the prothrombinase actions of factor Xa. It is interesting to note that the receptor may possess a calcium dependent second messenger pathway and that factor Xa has a mitogenic action for smooth muscle cells, as a result of Xa receptor activation or through the epidermal growth factor domain of this molecule.¹³

The thrombin receptor, a G protein coupled receptor, is widely distributed¹⁴ and its identification on tumour cells is of particular interest. Thrombin is known to play an important role in tumour biology, but the mechanisms of its action are less well understood. In experimental animal models and in vitro studies, pretreatment of tumour cells with thrombin enhances both metastatic potential in vivo and tumour cell adhesion in vitro.¹⁶ Other studies have shown that the specific thrombin inhibitor hirudin can dramatically reduce experimental metastasis of B16 melanoma cells in the nude mouse model.¹⁵ Stimulation of prostatic tumour cells with thrombin in culture leads to increased secretion of urokinase, an important enzyme involved in extracellular matrix remodelling and tumour invasion.⁶ These events may be mediated through the thrombin receptor. The fact that all three antigens are colocalised means that an efficient cell surface mechanism exists for the generation of thrombin in tumour cells.

Beginning with tissue factor and ending with thrombin receptor stimulation, this cascade may represent an important new autocrine mechanism in tumour cells. Interference with this pathway at any level may provide new therapeutic advances, both in modulation of tumour metastasis and in the prevention of

the thrombotic complications of cancer. It is already recognised that perioperative anti-thrombotic therapy for cancer surgery reduces late death from malignant disease¹⁷ and novel specific antithrombins may have superior effects.

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