Enhanced Expression of Urokinase Receptor Induced through the Tissue Factor-Factor VIIa Pathway in Human Pancreatic Cancer

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ABSTRACT

Overexpression of tissue factor (TF) is characteristically observed in advanced pancreatic cancer and has been associated with invasion and metastasis. Functional responses of TF activation are here investigated using as a model system the human pancreatic cancer cell lines SW979 (which overexpresses TF) and MIAPaCa2 (which does not express detectable levels). After stimulation of these cell lines with factor VIIa (FVIIa), the only known TF ligand, expression of urokinase receptor (uPAR) gene was up-regulated in SW979 cells in a dose-dependent manner but not in MIAPaCa2 cells. Interestingly, urokinase (uPA) and its specific inhibitor PAI-1 were not up-regulated. Exposure to functionally inactivated FVIIa did not show any effect on uPAR expression on SW979 cells despite binding to TF with higher efficiency. The neutralizing anti-TF antibody 5G9 blocked the FVIIa-induced up-regulation of uPAR completely, whereas hirudin failed to block this up-regulation. Treatment of SW979 cells with Factor Xa did not up-regulate the expression of uPAR gene, whereas treatment with FVII induced the same level of enhanced uPAR gene expression as that with FVIIa. In the matrigel invasion assay, enhanced invasion of SW979 cell line induced by FVIIa was completely inhibited by anti-TF antibody and α2-antiplasmin. Moreover, the endogenous levels of uPAR gene expression were significantly correlated with the level of TF gene expression in 19 human cancer cell lines (P < 0.05). These data suggest that up-regulation of uPAR expression by tumor cells leading to tumor invasion is induced through the TF-FVIIa pathway rather than TF-initiated thrombin generation. This is the first report that TF may be one of the key receptors that can up-regulate expression of the plasminogen activator receptor in human cancer cells to enhance tumor invasion and metastasis.

INTRODUCTION

TF is a transmembrane glycoprotein that can activate the coagulation zymogen FVII to the activated form FVIIa. The TF/FVIIa complex initiates the coagulation cascade leading to thrombin generation. TF is also a genuine receptor inducing an intracellular signal upon binding of its specific ligand FVII. It has been reported that TF is highly expressed in cancer cell lines that show enhanced metastatic potential, and it is also involved in the regulation of tumor growth (1, 2). Most colorectal cancer cell lines express TF, and the sublines established from metastatic lesions express higher cellular TF activity than the parental lines (3). Functional inhibition of TF using an anti-TF antibody results in a significant reduction of the number of metastatic lesions. However, thus far the mechanism of the increased metastatic potential induced by TF expression or activation is still unclear.

TF is a member of the class II cytokine receptor superfamily that includes the IFN α/β and γ receptor (4, 5). The cytoplasmic domain of TF is phosphorylated by a protein kinase C-dependent mechanism in response to phorbol 12-myristate 13-acetate (6). TF mediates a cytosolic Ca2+ signal upon interaction with FVIIa in J82 cells (7). Recently, several reports suggested that the binding of FVIIa to cell surface TF may induce transcriptional signaling. The present studies have been carried out to examine the hypothesis that the binding of FVIIa to TF may trigger the selective expression of genes that are involved in the invasive phenotype of cancer cells.

To identify alterations in gene expression in human pancreatic cancer cell lines upon binding of FVIIa to TF, we have studied expression of the components of the plasminogen activator system, which regulates fibrinolysis, by Northern blot analysis. uPA plays an important role in pericellular proteolysis during cell migration and tissue remodeling by physiological activation of plasminogen to plasmin. Binding of uPA to its receptor, uPAR, accelerates uPA activation from an enzymatically inactive proenzyme (pro-uPA). Significantly, overexpression of uPAR increased invasion by facilitating matrix degradation in a human osteosarcoma cell line (8). In human bladder cancer cell lines, both uPAR expression and uPA expression are required for cell invasion (9). The expression of genes of the plasminogen-activator system is differentially regulated by inhibitors of protein synthesis and butyrate (10–12).

In the present study, we found that there was selective overexpression of the uPAR gene induced by FVIIa binding to cell surface TF in human pancreatic cancer cells. Moreover, the endogenous levels of uPAR gene expression were correlated with the expression of TF gene in 19 human cancer cell lines. We believe that these observations suggest a mechanism by which TF is implicated in tumor invasion and metastasis.

MATERIALS AND METHODS

Materials. FVIIa and FVIIa were provided by Prof. E. G. D. Tuddenham (Medical Research Council Clinical Sciences Center, Imperial College School of Medicine, Hammersmith Hospital, London). FVIIa was derived by the blocking of FVIIa in the active site with N-Phe-l-Phe-l-Arg chloromethyl ketone as described previously (13). Recombinant hirudin and α2-AP were purchased from Sigma-Aldrich Company Ltd. (Poole, UK). Murine antihuman TF mAb 5G9 and 10H10 were provided by Dr. W. Ruf (Scripps Research Institute, La Jolla, CA; Ref. 14). Mouse antihuman uPAR mAb was provided by Dr. F. Lupu and Dr. V. Ellis (Thrombosis Research Institute, London).

Cell Lines. The 9 human pancreatic cancer cell lines and 10 human breast cancer cell lines used in this study were obtained from Imperial Cancer Research Fund Cell Production Services (Clare Hall Laboratories, London, UK). MIAPaCa2, ASPC1, MCF7, T47D, and ZR75-1 were cultured in RPMI 1640 supplemented with 10% FCS and 20 mm L-glutamine in a humidified incubator with 5% CO2 in air at 37°C. BxPC3, Capan2, T3M4, CTPlac1, SW850, SW979, Panc1, BT-7, BT-20, MDA-MB231, MDA-MB453, and MDA-MB468 were cultured in E4 medium with 10% fetal bovine serum in a humidified incubator with 10% CO2 in air at 37°C. MDA-MB415 and MDA-MB436 were cultured in E4 medium supplemented with 10% fetal bovine serum and 10 μg/ml insulin.
Flow Cytometry. MIAPaCa2 and SW979 cells were dissociated into single-cell suspension in Ca²⁺/Mg²⁺-free PBS. Cells were then pelleted by centrifugation and washed with ice-cold PBS with 0.1% (w/v) sodium azide (FACS washing buffer) twice. Cells were pelleted again, resuspended in FACS washing buffer with the primary antihuman TF mAb (10H10) at 50 μg/ml for 30 min, washed twice, and then incubated with FITC-conjugated sheep antimouse IgG (Sigma-Aldrich Ltd., Poole, UK) for 30 min. Cells were then washed with FACS washing buffer three times and resuspended in 500 μl of FACS washing buffer. Cell-associated fluorescence was quantified by flow cytometry with the FACS Vantage flow cytometer (Becton Dickinson Labware, San Jose, CA).

Northern Blot Analysis. The cells were plated at a density of 10⁶ cells/100-mm dish and grown to 70–80% confluence in medium supplemented with 10% FCS. Before being used for stimulation experiments, cells were cultured in medium supplemented with 0.5% FCS for 48 h. Total cellular RNA samples were isolated from cells using QIAGEN RNaseasy kit (QIAGEN Ltd., UK) according to the manufacturer’s protocol. For Northern transfer, 10 μg of each RNA sample was denatured, subjected to electrophoresis in 1.2% formaldehyde-agarose gel, and transferred to a Hybond-N+ nylon membrane (Amersham Life Sciences, Amersham, UK) by capillary transfer. After transfer, RNA was cross-linked onto the membrane by UV irradiation. Hybridization was performed in Rapid-hyb buffer (Amersham RPN 1636) and heat-denatured herring sperm DNA at 64°C for 2 h. The membranes were washed twice with 2 × SSC-0.1% SDS at 64°C, then once with 1 × SSC-0.1% SDS at 64°C. The membranes were autoradiographed against Amersham Hyperfilm at −80°C for 2 to 5 days. The human cDNA probes of uPA (600-bp fragment cut by EcoRI/HindIII), uPAR (1113-bp fragment cut by XbaI/EcoRI), and PAI-1 (504-bp fragment cut by PstI), which were subcloned into pBluescript M13+, were kindly provided by Dr. M. F. Scully (Thrombosis Research Institute, London). Probes were radiolabeled with [α-32P]dCTP (Amersham) using a DNA Labeling Kit (-dCTP; Pharmacia Biotech). Relative signal intensity was determined on a Molecular Imager (Bio-Rad Laboratories, CA94547) using Molecular Analyst Software Version 2.1.

Western Blot Analysis. Cells were washed with ice-cold Ca²⁺/Mg²⁺-free PBS, then treated with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 1 μg/ml aprotinin, and freshly added 100 μg/ml phenylmethylsulfonyl fluoride). The lysate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and electrophoresed on a 10% SDS-polyacrylamide gel, followed by transfer of the proteins onto Hybond™ ECL™ Nitrocellulose membrane (Amersham) using a semi-dry transfer system. The blots were incubated with antihuman uPAR mAb, and bands were detected using the ECL Western blotting system (Amersham) according to the manufacturer’s protocol.

Matrigel Migration Assay. SW979 migration was studied using the Transwell Cell Culture Chamber (12-mm diameter, 12-μm pore size) purchased from Costar (Cambridge, MA). The chambers were coated with Matrigel (Becton Dickinson Labware, Oxford, UK) that was diluted with 2 volumes of OPTI-MEM (Life Technologies Ltd., Paisley, UK). Two × 10⁵ cells were seeded with 500 μl of medium supplemented with 0.5% FCS and incubated in 10% CO₂ at 37°C for 72 h; then the matrigel on the upper surface of the filters was removed. The filters were fixed with 100% methanol and stained with H&E. The number of cells that had migrated to the lower surface was counted in six random fields using a light microscope (×400).

Statistical Analysis. Statistical analysis of matrigel migration assay was performed by using ANOVA and unpaired Student’s t test. The Kruskal-Wallis test was done for determination of correlation of gene expression. All statistics were performed in two-sided tests. P < 0.05 was considered significant.

RESULTS

In Northern blot analysis, TF mRNA, especially the 2.2- and 3.1-kb species, was highly expressed in SW979, T3M4, ASPC1, and Cfpac1 cell lines, moderately expressed in BxPC3 and SW850 cell lines, but not expressed in MIAPaCa2, Panc1, and CaPan2 cell lines (Fig. 1A). In FACS analysis, TF antigen was highly expressed in the SW979 cell line but not expressed in the MIAPaCa2 cell line (Fig. 1B). This result was consistent with the results of Northern blot analysis.

Cell-specific expression of uPAR, uPA, and PAI-1 mRNA induced by FVIIa was examined by Northern blot analysis. In SW979 cells that highly express TF cell surface receptor, uPAR mRNA was increased after treatment with FVIIa in a dose-dependent manner, whereas uPA and PAI-1 mRNA were not increased (Fig. 2A). In the analysis of relative signal intensity using a Molecular Imager, expression of uPAR gene was approximately 2.7-fold increased by treatment with 100 nM FVIIa compared to control. The affinity of TF for FVIIa is known to be in the nanomolar range from previous studies (13). This up-regulation of uPAR mRNA was not found after treatment with FVIIai, an antagonist of FVIIa under the same conditions (Fig. 2A). In the MIAPaCa2 cell line, which does not express detectable levels of TF, up-regulation of uPAR mRNA was not observed after treatment with FVIIai (Fig. 2B). In time course studies, uPAR expression was maximally increased to 5-fold over baseline 2–4 h after treatment with 100 nM FVIIa and decreased to the endogenous level by 48 h after treatment with FVIIa in SW979 cells (Fig. 3A). In contrast, levels of uPA, PAI-1 3.4-kb and PAI-1 2.2-kb transcription were increased less than 1.3-fold 2–4 h after treatment. We also performed Western blotting with uPAR mAb, which confirmed that the expression of protein (Mr 60,000) was increased compared to control after incubation with FVIIa for 3–24 h (Fig. 3B).

Incubation of SW979 cells with the neutralizing anti-TF mAb 5G9 resulted in complete inhibition of uPAR gene expression induced by FVIIa, whereas the nonneutralizing anti-TF mAb, 10H10, had no effect on uPAR gene up-regulation (Fig. 4). These results suggest that the binding of FVIIa to TF on cell surface is necessary to induce the gene up-regulation.
To determine whether this up-regulation of uPAR mRNA was induced by factor X activation or thrombin generation triggered by the FVIIIa-TF complex, the cells were treated with FXa. Treatment of SW979 cells with FXa did not up-regulate the expression of uPAR gene, while treatment with FVII induced the same level of uPAR gene expression as that with FVIIa (Fig. 5). The expression of uPAR induced by treatment with FVIIa was not affected by preincubation with the thrombin inhibitor, hirudin (Fig. 6). Treatment with a-thrombin or hirudin itself caused slightly (approximately 1.3-fold) increased expression of uPAR mRNA compared to control in SW979 cells. This effect was not significant compared to FVIIa treatment. These data indicate that neither factor X activation nor thrombin generation are implicated in the specific up-regulation of uPAR gene on SW979 cells, but the active-site of FVIIa might be important for up-regulation of the uPAR gene through the TF-FVIIa pathway.

To examine whether the specific up-regulation of uPAR induced by the treatment with FVIIa is associated with an invasive phenotype, the matrigel migration assay was performed. The migration of SW979 cells was increased by treatment with FVIIa in a dose-dependent manner, but not increased by FVIIa (Fig. 7A). This increased migration was completely inhibited not only by the neutralizing anti-TF mAb, 5G9, but also by the specific plasmin inhibitor, α2-AP (Fig. 7B). However, hirudin did not inhibit the increased migration mediated by FVIIa-TF complex. Moreover, hirudin itself enhanced the cell migration to some degree in our
the thrombin receptor on malignant melanoma cells, although there is another report that suggested that the metastatic effect induced by TF expression did not involve products of the coagulation cascade (2); or (b) binding of FVIIa to TF may directly induce signals that are relevant to the metastatic tumor phenotype. Recently, Pendurthi et al. (16) used the differential display technique to show up-regulated transcription of poly(A)polymerase gene induced in human fibroblast cells by incubation with FVIIa. Poulsen et al. (17) demonstrated mitogen-activated protein kinase (MAPK) activation on exposure of baby hamster kidney cells to FVIIa that could not be blocked by tick anticoagulant protein and was not seen on exposure to FXa. This evidence supports the possibility that TF-mediated signal transduction events may involve up-regulation of cancer-related genes.

During cell migration, expression of the plasminogen activator system is up-regulated in several types of cells. uPAR is attached to the cell membrane by a glycosyl phosphatidyl inositol anchor that is added during posttranslational processing and that also involves COOH-terminal truncation of the primary translation product (18, 19). Binding of uPA to uPAR accelerates uPA activation from an enzymatically inactive proenzyme (pro-uPA). uPAR is also reported to be a genuine receptor that induces intracellular signal transduction events that lead to tumor cell activation. Soluble uPAR variants, which do not have the GPI anchor, have been reported (20, 21). uPA and uPAR are usually found at the leading cell edge at which plasminogen activity is accumulated to facilitate migration (22, 23). Interactions of PAI-1 and uPAR with the extracellular matrix protein vitronectin (VN) and integrin receptors have been reported (24–26). Overexpression experiments. These results suggest that the specific up-regulation of uPAR induced by the treatment with FVIIa could increase SW979 cell migration in vitro.

To compare endogenous expression levels of TF gene and uPAR, uPA and PAI-1, total RNA was extracted from 9 pancreatic cancer cell lines and 10 breast cancer cell lines that were cultured in medium with 10% FCS. We found that there was significant correlation between the expression levels of TF mRNA and uPAR mRNA (P < 0.05) in these 19 human cancer cell lines, whereas the expression of neither uPA mRNA nor PAI-1 mRNA was correlated with the expression of TF mRNA (Table 1).

DISCUSSION

Several recent studies have suggested the involvement of TF overexpression in the metastatic tumor phenotype (1–3), but thus far there have not been any reports that could explain how TF could influence invasive properties. There are two broad possibilities to explain this issue: (a) TF-FVIIa complex-driven initiation of the coagulation might activate a downstream-proteinase activated receptor or the thrombin receptor. Fisher et al. (15) showed that TF-initiated thrombin generation activated signaling through
hances migration not only on tumor cells (8, 9) but also on endothelial cells (32). In the Matrigel cell migration assay, the migration of SW979 cells was increased by incubation with FVIIa in a dose-dependent manner, and FVIIa-induced cell migration was completely inhibited by the neutralizing anti-TF mAb 5G9. To confirm whether the FVIIa-induced cell migration is caused by the enhanced expression of uPAR that binds endogenous uPA on the cell surface and enhances plasmin generation to mediate degradation of the Matrigel, cells were incubated with FVIIa in the presence of the specific plasmin inhibitor α2-AP. FVIIa-induced cell migration was reduced to basal levels by coincubation with α2-AP. In contrast, cell migration was increased by coincubation with FVIIa in the presence of hirudin. These results are consistent with the evidence of enhanced uPAR expression induced by the TF-FVIIa pathway leading to activation of the plasminogen activator system to facilitate cell invasion.

We also demonstrated that endogenous expression levels of TF were significantly correlated with those of uPAR in 19 human cancer cell lines ($P < 0.05$). This result suggests that not only binding of FVIIa to TF but also expression of TF itself may be important for the regulation of uPAR gene expression. In recent studies, Carmeliet et al. (33) demonstrated that inactivation of the TF gene resulted in the failure of blood vessel formation leading to embryonic death in knockout mice. Rosen et al. (34) showed that mice lacking FVIIa developed normally but suffered fatal perinatal bleeding. Hence, expression of TF appears to be crucial for vessel development, and FVIIa plays an important role in sustaining TF function for mainte-
nance of vessels. In terms of the contributions of TF expression and FVIIa binding in influencing gene expression, our new evidence may be consistent with these observations.

Recently several reports demonstrated an association between TF expression and tumor grade. Kakkar et al. (35) showed that TF is not expressed in normal pancreatic tissue, whereas in pancreatic carcinoma, expression increases with progressive tumor dedifferentiation. In this study, 6 of 9 pancreatic cancer cell lines and 5 of 10 breast cancer cell lines expressed significant levels of TF mRNA. Clinically, pancreatic cancer is associated with a high frequency of thrombotic complications that may result from activation of the extrinsic pathway of blood coagulation and higher circulating levels of FVIIa (36). From this aspect, TF could be one of the most attractive targets for cancer therapy using not only anti-TF antibody to induce tumor vascular thrombosis (37) but also gene therapy aimed at down-regulation of TF gene to prevent tumor metastasis.

In conclusion, we have demonstrated that the binding of FVIIa to TF up-regulates uPAR expression and results in increased tumor migration by pancreatic cancer cells. Moreover, increased TF expression on the cell surface is associated with elevated expression of uPAR in human cancer cell lines. This is the first report to propose a potential molecular mechanism of TF-FVIIa complex-mediated signal transduction that can explain TF-induced tumor invasion and metastasis.

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