Stromelysin 3 is overexpressed in human pancreatic carcinoma and regulated by retinoic acid in pancreatic carcinoma cell lines

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Abstract

Background—Matrix metalloproteinases play an important role in the control of local tumour growth and metastasis of human pancreatic cancer.

Aims—To examine expression of recently discovered stromelysin 3 (STR-3) in human pancreatic cancer and pancreatic carcinoma cell lines and to investigate their regulation by retinoids.

Methods—STR-3 expression was examined by immunohistochemistry in 21 human pancreatic carcinomas. Expression of STR-3 and regulation by retinoids was assessed in five human pancreatic carcinoma cell lines using western and northern blotting as well as nuclear run on assays.

Results—There was pronounced overexpression of STR-3 in 17 of 21 (80.9%) pancreatic carcinoma specimens. STR-3 expression was predominantly located in peritumourous stromal cells. Six of 21 (28.5%) carcinomas also revealed STR-3 expression in epithelial tumour cells whereas no STR-3 expression was observed in non-transformed pancreas. All five pancreatic carcinoma cell lines expressed STR-3 mRNA and protein. Furthermore, retinoid treatment results in a time and dose dependent inhibition of STR-3 protein expression. This inhibition seems to be post-transcriptional as neither STR-3 gene transcription nor mRNA steady state concentrations were affected by retinoids.

Conclusions—STR-3 overexpression in stromal as well as epithelial elements during pancreatic carcinogenesis might contribute to the aggressive local growth and metastasis of pancreatic cancer and can be therapeutically targeted by retinoids.

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Keywords: matrix metalloproteinases; pancreatic cancer; stromelysin 3; retinoic acid

The prognosis of pancreatic cancer is extremely poor with an estimated five year survival rate of less than 2%. Surgery being the only potential cure, the prognosis of these patients is critically determined by the extent of local tumour growth, peritumourous invasion, and the presence of distant metastases.

Cancer cells can be distinguished from their non-transformed counterparts by their ability to invade normal tissue and metastasise via lymphatics and blood vessels. These processes are critically orchestrated by matrix metalloproteinases (MMPs); this enzyme family is capable of degrading collagenous and non-collagenous components of the extracellular matrix, thereby promoting tumour cell invasion, metastasis, and neovascularisation.

Recently, a novel matrix metalloproteinase, designated stromelysin 3 (STR-3 or MMP 11), has been described in human breast cancer. Due to its unique expression pattern and its substrate specificity, this enzyme has attracted great interest. STR-3 expression has been tightly linked with the extent of local invasiveness of breast, head and neck, as well as basal skin cancers, implicating its important function in multiple epithelial malignancies. Further supporting its role in local invasiveness and tumour spread, overexpression of STR-3 occurs in lymph nodes as well as in distant metastatic sites of the primary tumours.

In the case of breast carcinomas, elevated STR-3 mRNA levels in the primary tumour are highly predictive for the presence of distant metastases. Similarly, in the case of cervical carcinoma, STR-3 expression correlates with the degree of malignant transformation: although no STR-3 expression is observed in normal cervical mucosa, it can be detected in approximately 50% of dysplasias, 70% of carcinomas in situ, and 100% of invasive carcinomas. Although overexpressed in a variety of epithelial malignancies, STR-3 synthesis has so far been exclusively described in mesenchymal cells of the tumour stroma.

In addition to its restricted expression pattern, STR-3 reveals specific biochemical features which allow clear distinction from other previously described MMPs. For example, unlike other MMPs, STR-3 is processed by furin to its enzymatically active form within the constitutive secretory pathway and is therefore secreted as an active enzyme.

In addition, STR-3 displays a distinct pattern of substrate specificity which includes serine protease inhibitors (serpins) such as the α-1 proteinase inhibitor (α-1 antitrypsin). Enzymatic degradation of α-1 antitrypsin by STR-3 seems of particular interest because α-1 antitrypsin has been shown to be a potent inhibitor of anchorage independent growth in epithelial carcinoma cells.

Furthermore, α-1 antitrypsin functions specifically as an inhibitor of leucocyte elastase, a tissue destructive proteinase stored in neutrophils and monocytes. Taking these observations together, these data suggest that STR-3 dependent inactivation of α-1 antitrypsin simultaneously affects the proliferative and invasive activity of neoplastic cells.
Therefore, based on evidence for its regulatory function in the control of local tumour growth and invasiveness, the current study was designed to evaluate the expression and regulation of STR-3 in a panel of human pancreatic carcinomas and cell lines.

Materials and methods

Materials

Human pancreatic carcinoma cell lines AsPc1, Capan 1, Capan 2, and Panc-1 were obtained from the American Type Tissue Culture Collection. The cell line Dan-G was obtained from the Deutsches Krebsforschungszentrum (Heidelberg, Germany). Dulbecco’s modified Eagle medium was obtained from Gibco (Berlin, Germany), and fetal calf serum from Biochrom (Berlin, Germany). All retinoids were kindly provided by Hoffmann-La Roche (Basel, Switzerland). α-32P deoxycytidine triphosphate (6000 Ci/mmol) was obtained from DuPont (Bad Homburg, Germany). The random priming labelling kit was obtained from Amersham (Braunschweig, Germany); nitrocellulose and blotting paper were purchased from Schleicher and Schuell (Dassel, Germany); Poly AdTract isolation kit was obtained from Serva (Heidelberg, Germany); RNA molecular size markers and random hexamer primers were from Bethesda Research Laboratories (BRL, Bethesda, Maryland, USA); and restriction enzymes were from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade and purchased from Sigma (Deisenhofen, Germany).

Cell culture

All cell lines were grown as subconfluent monolayer cultures supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 U/ml). The cells were kept under 95% air and 5% CO2 at 37°C. All experiments were performed in the log phase of growth after the cells had been plated for 24 hours unless otherwise stated. Retinoids were prepared under subdued light as stock solutions in ethanol and aliquots were kept stored at −20°C until used. Retinoids were added to the cultures from stock solutions. Control cells were employed. Nuclei were either used fresh or after freezing at −80°C until use. RNA products were purified using DNase, proteinase K in the presence of SDS, and salt precipitation according to the procedure of Nelson and Groudine. Unincorporated nucleotides were removed by passing the reaction sample over a Sephadex G 50 column (Sigma, Deisenhofen, Germany). The specific activity of the probes was routinely 1–2 × 107 cpm/μg DNA. Filters were prehybridised and hybridised as previously described. Following hybridisation, filters were sequentially washed with increasing stringency as previously described. Filters were blotted dry and exposed to x-ray film for one to five days using two intensifying screens. The detected signals were quantitated by laser densitometry.

Nuclear transcription assay

Nuclei were isolated by sucrose gradient centrifugation from cells incubated in the absence or presence of retinoids as previously described. In each reaction 5 × 109 nuclei were employed. Nuclei were either used fresh or after freezing at −80°C until use. RNA products were purified using DNase, proteinase K in the presence of SDS, and salt precipitation according to the procedure of Nelson and Groudine. Unincorporated nuclei were removed by centrifugation over a spin column. Routinely, 3–10 × 106 cpm were added to each experimental condition. Care was taken that each experimental condition contained the same amount of nuclei as well as the same amount of radioactivity. A 4 μg aliquot of each cDNA was denatured by heating to 65°C for one hour in 0.2 M NaOH and applied to nitrocellulose using a slot blot manifold. The filters were prehybridised and hybridised as previously described. Following hybridisation, the filters were washed twice in 40 mM nitrocellulose membranes. Non-specific binding was blocked by 5% (wt/vol) fat-free milk solution. Blots were then allowed to react with a mouse monoclonal antibody directed against human STR-3 (5ST-4A9, kind gift of P Bass, IGBMC, Illkirch, France) at a final dilution of 1/3000 overnight. After extensive washing, blots were incubated with goat antimouse IgG conjugated to alkaline phosphatase as a second antibody. Protein bands were subsequently detected using alkaline phosphatase dependent nitroblue tetrazolium/bromochloroindoylphosphate reduction. The detected signals were quantitated by laser densitometry.
Sodium phosphate at 42°C for 10 minutes and then exposed to x-ray films for 24 hours. In one set of nuclei, α-amanitin was added at a concentration of 2 µg/ml to verify the specificity of the observed hybridisation signal.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Immunohistochemical analysis of surgically resected formalin fixed, paraffin wax embedded pancreatic cancer tissue samples was carried out using the alkaline phosphatase/antialkaline phosphatase (APAAP) method, after pressure cooker antigen retrieval. Rehydrated sections were placed in a pressure cooker filled with 10 mM citrate buffer (pH 6.0) and allowed to boil for two minutes. Sections were incubated at room temperature overnight with mouse monoclonal antibody 5ST-4A9 at a final dilution of 1/6000. The sections were treated for 30 minutes with rabbit antimouse antibody, followed by treatment for 30 minutes with the APAAP complex. The secondary antibody and the APAAP complex were applied twice for another 10 minutes and new fuchsin was used as a developer. The sections were then counterstained with haemalum.

Three independent approaches were used to confirm the specificity of the observed immunohistochemical signal: serial dilution of the primary antibody until the signal disappeared; irrelevant first monoclonal antibody against human albumin; and preimmune mouse IgG, all of which failed to reveal relevant staining. The

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Tumour differentiation was graded according to Klöppel’s criteria. NP, not present.

**Figure 1** STR-3 expression in normal pancreas and pancreatic carcinoma. No specific staining was observed in mesenchymal or epithelial cells of non-transformed pancreas (A,B). STR-3 was overexpressed by stromal cells in pancreatic carcinoma showing strongest expression in stromal cells directly surrounding tumour cells (C,D). STR-3 expression was also observed in pancreatic carcinoma epithelial cells in 6/21 cases (E,F).
slides were analysed by two independent investigators. The degree of tumour differentiation was determined following the criteria of Klöppel.21

Results

EXPRESSION OF STR-3 IN HUMAN Pancreatic Carcinomas

Using immunohistochemistry with a monoclonal antibody specific for human STR-3, we analysed 21 surgically resected pancreatic adenocarcinomas, nine of which contained non-transformed pancreatic tissue at the resection margins. STR-3 was detected in neither mesenchymal nor epithelial cells of the non-transformed pancreas (n=9) (fig 1 and table 1). In contrast, 17 of 21 carcinomas (80.9%) revealed a strong immunohistochemical signal for STR-3 located in the stromal cells of the tumour (fig 1). Consistent throughout all examined tumour samples, STR-3 expression was strongest in the stroma cells directly surrounding pancreatic cancer cells and weaker in stroma cells distant to the primary tumour (fig 1C,D). In addition, we observed STR-3 expression in six of 21 carcinomas (28.5%) in ductal epithelial tumour cells (fig 1E,F), albeit consistently at a lower degree than in the mesenchymal cells of the corresponding carcinoma. Negative controls using irrelevant first antibody, serial dilution of anti-STR-3 antibody, or preimmune mouse IgG were consistently negative (data not shown). Although all of the poorly differentiated adenocarcinomas expressed STR-3 protein in their stromal compartment, there was no significant correlation between the degree of tumour differentiation and the extent of STR-3 expression (table 1). Furthermore, except for case 1, all tumours that stained positively for STR-3 in the epithelial cancer cells also expressed STR-3 in the corresponding mesenchyme (table 1).

EXPRESSION OF STR-3 IN HUMAN PANCREATIC Carcinoma cell lines

Based on our immunohistochemical results suggesting STR-3 expression in approximately one third of pancreatic epithelial cancer cells, we were interested to establish an in vitro system which would allow study of the regulation of STR-3 gene expression. Using a human STR-3 cDNA probe in northern blot analysis, we detected a single 2.4 kb mRNA transcript corresponding to STR-3 in all cell lines tested (fig 2B). To determine whether the expression of STR-3 mRNA results in synthesis and secretion of the corresponding protein, we performed western blot analysis using a mouse monoclonal antibody specific for human STR-3 of supernatant proteins in the same panel of cell lines. As shown in fig 2A, a single band of approximately 50 kDa corresponding to the active form of STR-3 was detected in the supernatant of all five cell lines. To our knowledge, this represents the first in vitro system of...
epithelial cell lines expressing STR-3 mRNA and protein.

REGULATION OF STR-3 EXPRESSION BY RETINOIDS

We have previously shown that treatment with retinoids results in inhibition of growth, induction of differentiation, and a decrease in the metastatic potential of human pancreatic carcinoma cells in vitro and in vivo.18 22 We therefore extended these observations and examined whether retinoids might also be capable of modulating STR-3 expression. Incubation of pancreatic carcinoma cell lines with 10 µM 13-cis-retinoic acid resulted in a profound decrease in STR-3 protein expression in AsPc1, Capan 2, Dan-G, and Panc-1 cells (fig 3). The degree of retinoid mediated inhibition varied between 14% of control in Capan 2 cells and 34% of control in Panc-1 cells after 48 hours of retinoid treatment.

Using the Dan-G cell line as a representative in vitro model we further characterised the retinoid mediated effects in more detail. The inhibitory effects of 13-cis-retinoic acid on STR-3 expression occurred in a time and dose dependent manner. After 72 hours of retinoid incubation, STR-3 concentrations were maximally inhibited to 4 (2)% of untreated controls (n=3) (fig 4). Extended incubation did not result in a further decrease (data not shown). Half maximal inhibition occurred at 5 µM and maximal inhibition at 10 µM 13-cis-retinoic acid, whereas concentrations lower than 1 µM were ineffective (fig 5). We next investigated the relative biological activity of various physiologically occurring human retinoids. Incubation with 10 µM of the indicated retinoid for 48 hours revealed that 13-cis- and all-trans-retinoic acid were equally potent in terms of inhibiting STR-3 expression, whereas the stereoisomer 9-cis-retinoic acid had no significant effect on STR-3 expression in Dan-G cells (82 (10)% of control, n=3) (fig 6).

Discussion

The fate and prognosis of patients with pancreatic cancer depends mainly on the extent of local tumour invasion, lymph node involvement, and the presence of distant metastases. Expression of the matrix metalloproteinase stromelysin-3 has been tightly linked specifically to these pathophysiological features in carcinomas of the breast and head and neck, as well as basal skin cancers.7–10 There are currently no experimental data available as to the expression of STR-3 in human pancreatic malignancies. Furthermore,
given the lack of effective chemotherapy in advanced pancreatic carcinoma we are interested to identify novel targets for an experimental treatment strategy using retinoids in pancreatic cancer.18 22 Our study was therefore designed to explore the expression and retinoid mediated regulation of STR-3 in human pancreatic carcinomas. Several interesting and novel observations can be deduced from this study.

By immunohistochemistry we did not observe STR-3 expression in epithelial or stromal cells of the non-transformed, normal human pancreas. In contrast, a high percentage of pancreatic adenocarcinomas (more than 80%) expressed significant amounts of STR-3. This high percentage of STR-3 positive pancreatic tumours is similar to what has been observed in breast as well as head and neck cancer.7 12 but different to renal cell carcinoma and malignant melanoma, where STR-3 expression is observed in only 4% and 27% of all cases, respectively.12 Therefore, STR-3 displays a tissue specific and restricted expression pattern in human malignancies. As in breast as well as head and neck cancer, there was no obvious correlation between the degree of tumour differentiation and the presence or extent of STR-3 expression.11 In all cases examined, STR-3 expression was predominantly located in the stromal cells directly surrounding the tumour, suggesting that stimulatory factors released by pancreatic carcinoma cells induce STR-3 expression in the adjacent mesenchymal cells. This hypothesis is further supported by in vitro studies in fibroblasts, showing that basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet derived growth factor (PDGF), all of which are overexpressed by pancreatic carcinoma cells,23 24 act as potent inducers of STR-3 expression.25

To our surprise, we also detected STR-3 in pancreatic epithelial tumour cells in approximately one third of the examined cases. This observation was further supported by in vitro experiments, showing specific STR-3 mRNA and protein expression in all five human pancreatic carcinoma cell lines. To our knowledge, this represents the first evidence that STR-3 can be expressed by epithelial cells in vivo and in vitro. Although a large variety of tissues and cell lines have been examined, so far STR-3 expression has been exclusively described in mesenchymal cells.27 Even in breast carcinoma, where almost 100% of all tumours overexpress STR-3, no such expression could be observed in epithelial tumour cells or a broad panel of breast carcinoma cell lines.7 Given the potential role of STR-3 in the regulation of local tumour growth and metastasis, these findings suggest that in human pancreatic cancer tumour cells, the surrounding stromal cells might also contribute to the invasive phenotype by synthesising and secreting STR-3 on malignant transformation.

The second rationale of this study was to analyse the retinoid regulated expression of STR-3 in pancreatic carcinoma cells. This is based on our previous observation that retinoid treatment results in growth inhibition, induction of cellular differentiation, and a decreased invasive capacity of human pancreatic carcinoma cells in vitro and in vivo.18 22 These observations make retinoids an attractive experimental therapeutic approach for the treatment of advanced human pancreatic carcinoma. We therefore examined whether retinoid mediated down regulation of tumour cell derived STR-3 expression might add another therapeutically beneficial aspect to this concept. Our observation of a panel of five human pancreatic carcinoma cell lines expressing STR-3 mRNA and protein therefore provides the first suitable in vitro system to study retinoid regulation of STR-3 expression in human epithelial tumour cells. Unlike other matrix metalloproteinases, the STR-3 protein contains a unique 10 amino acid insert between the pro and catalytic domains that includes a recognition motif for the Golgi associated protein convertase, furin.13 Consequently, within the constitutive secretory pathway, STR-3 is enzymatically processed to its active form and can be readily detected in the supernatant of STR-3 secreting cells. Incubation of pancreatic carcinoma cell lines with therapeutically achievable concentrations of 13-cis-retinoic acid,26 resulted in a pronounced decrease in STR-3 protein concentrations in four of five cell lines tested. Using the Dan-G cell line as a representative model, we found that the inhibitory effects of 13-cis-retinoic acid on STR-3 expression were time and dose dependent. When we tested three therapeutic relevant and clinically available retinoid analogues for their ability to inhibit STR-3 expression we found that on an equimolar basis all-trans- and 13-cis-retinoic acid were potent inhibitors of STR-3 expression whereas 9-cis-retinoic acid had no significant effect. This is in good agreement with our previous experiments, in which we showed that 13-cis-retinoic acid is the most biologically active retinoid in
terms of growth inhibition and induction of differentiation in human pancreatic cancer cells.14

Retinoids are believed to exert part of their biological effects via two families of nuclear retinoic acid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). On binding of their ligand, both receptor families act as ligand activated transcription factors thereby controlling gene transcription initiated from promoters of retinoid regulated genes.27 28 Along this line it has previously been shown that retinoic acid acts as a potent transcriptional inhibitor of other matrix metalloproteinases such as collagenase (MMP-1) and stromelysin.29 30 We therefore hypothesised that the retinoid mediated decrease of STR-3 expression might occur as a consequence of a decreased transcription rate of the STR-3 gene. However, when we assessed STR-3 gene transcription by means of nuclear run on assays, we repeatedly failed to detect a transcriptional effect of 13-cis-retinoic acid. Furthermore, as revealed by northern blot analysis, STR-3 mRNA steady state concentrations remained unchanged when Dan-G cells were incubated with retinoids for up to four days. These data therefore suggest that the retinoid mediated inhibition of STR-3 expression in pancreatic carcinoma cells occurs at a translational level. Alternatively, inhibition of cellular STR-3 secretion by retinoids could be accountable for the observable decrease in STR-3 concentrations after retinoid treatment. This hypothesis however seems unlikely on the basis of the following observations: STR-3 is secreted via the constitutive and not the regulated secretory pathway31; and using identical experimental conditions we observed that retinoid treatment results in an increase in MMP-1 concentrations in the supernatant of the same cell line, precluding a non-specific inhibitory effect on cellular secretion by retinoids (von Marschall et al, manuscript in preparation). Although retinoids have previously been shown to block the bFGF and PDGF mediated stimulation of STR-3 expression in human lung fibroblasts,32 this represents the first experimental evidence of a direct regulatory effect of retinoids on STR-3 expression.

In summary, in malignant transformation of the human pancreas, stromal elements as well as epithelial tumour cells contribute to the de novo expression of stromelysin-3, which can be therapeutically down regulated by retinoid treatment.

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