

The Expression of the Thrombin Receptors PAR-3 and PAR-4 is Downregulated in Pancreatic Cancer Cell Lines

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Abstract:

Background: Patients with pancreatic cancer frequently suffer from thrombosis as a consequence of excess thrombin generation. In addition to its role in the plasmatic coagulation cascade, thrombin induces numerous cellular effects by activating a unique group of G-protein-coupled receptors on the cell membrane, the proteinase-activated receptors (PARs). At present, PAR-1, PAR-3 and PAR-4 are known to be activated by thrombin. We previously demonstrated a putative role for PAR-1 in pancreatic cancer progression, but little is known about the physiological and pathophysiological roles of PAR-3 and PAR-4. In the present study, we examined the expression patterns of PAR-3 and PAR-4 in pancreatic tissue and pancreatic cancer cells.

Methods: Tissue samples from three patients with pancreatic adenocarcinoma and six human pancreatic carcinoma cell lines were examined. Gene expression was analysed by RT-PCR and quantified by HPLC. Protein expression was determined by Western blot analysis. Data analysis was performed using ANOVA in SPSS.

Results and Conclusion: In contrast to PAR-1, both PAR-3 and PAR-4 were expressed in healthy pancreases but downregulated in pancreatic cancer. The contrasting expression patterns of PAR-3 and PAR-4 compared with PAR-1 indicate that the mechanism that regulates the cellular effects of thrombin on tumor progression remains to be fully elucidated.

Keywords: Gene Expression; Pancreatic Cancer; Protein Expression; Thrombin; Thrombin Receptors

Introduction

Thrombosis is a common complication of cancer; it is the second most common reason for death after the progression of the tumor [1,2]. Professor Armand Trousseau first described thrombophlebitis migrans as the first presenting sign of a gastrointestinal malignancy in 1864 [3,4]. Among gastrointestinal tumors, pancreatic cancer has the highest rate of thromboembolic complications [5]. Up to 40% of pancreatic cancer patients display symptoms of deep vein thrombosis (DVT) [6–9].

The activation of the coagulation cascade leads to the excess generation of the serine protease thrombin, its key enzyme. Thrombin plays a central role in activating several coagulation factors and splitting fibrinogen into the active product fibrin [10].

In addition to its diverse plasmatic actions, thrombin elicits numerous cellular responses, such as platelet aggregation, the secretion of cytokines from endothelial cells and the proliferation of fibroblasts and smooth muscle cells [11–13]. Thrombin has also been proposed to activate/aggravate the oncogenic potential of both normal and malignant cells [14–16]. The cellular effects of thrombin are mediated by a subgroup of G-protein receptors, the protease-activated receptors (PARs) [17,18]. PAR-1, the first identified protease-activated

receptor [19,20], plays a crucial role in the activation of platelets and is thought to be the link between thrombin and the activation of the oncopathogenic potential in both benign and malignant cells via the aggravation of cell motility, tumor growth and angiogenesis [21–24].

We previously demonstrated poor PAR-1 expression in healthy pancreatic tissue and PAR-1 upregulation in pancreatic cancer tissue and pancreatic adenocarcinoma cell lines. The level of PAR-1 expression was inversely correlated with the grade of pancreatic cancer cells differentiation [25]. Furthermore, the activation of PAR-1 in pancreatic adenocarcinoma cell lines induced intracellular signalling, resulting in increased proliferation [26].

The recently cloned protease-activated receptors PAR-3 and PAR-4 also belong to the PAR family, but they have not been well characterised. Both PAR-3 and PAR-4 are specifically activated by thrombin [27–29], but their role in physiological and pathophysiological processes is not clearly understood.

The aim of this study was to determine the expression patterns of PAR-3 and PAR-4 in pancreatic cancer and to further characterise their putative role in thrombin-induced cancer progression.

Methods and Materials

Tissue samples

Tissue samples from three patients with pancreatic adenocarcinoma were obtained from the tumor and healthy surrounding tissue and intraoperatively cryoconserved. Written informed consent was obtained from all the patients prior to surgery.

Cell culture and reagents

The human pancreatic carcinoma cell lines MIA PaCa-2 [30] (ATCC, Rockville, MD, USA); PATU 8902 and PATU 8988s [31,32], Capan-1 and Capan-2 [33,34], and DAN-G [35] (all DSMZ, Braunschweig, Germany) and Capan-1 (a gift from Professor Marc M. Mareel, University of Gent, Belgium) were cultivated in their respective media at 37°C in a humidified atmosphere with 5% CO₂ (see also Table 1). Human Umbilical Vein Endothelial Cells (HUVECs) (Boehringer Ingelheim, Heidelberg, Germany), which express PAR-4 were included as positive controls for PAR-4 expression. The medium and supplements were purchased from Biochrom Seromed (Berlin, Germany). Cell culture plastic ware was obtained from Nunc AS (Roskilden, Denmark).

RNA isolation and reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from pancreatic tissue samples, pancreatic cancer cell lines and HUVECs using a high-purity RNA tissue kit and a high-purity RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

For RT-PCR analysis, 250 to 500 ng of total RNA from each pancreatic adenocarcinoma cell line or tissue sample was used in SuperScript™ One-Step RT-PCR with Platinum™ Taq (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions. Gene specific primer were designed using the web based primer blast software from ncbi (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The gene-specific primers for PAR-3 were as follows: sense 5'-CTG TTG CCC ACT TTT TGT CAG A -3' and antisense 5'-TGT TGC CCA CAC CAG TCC ACA -3'. The following PCR conditions were used to amplify PAR-3: 30 minutes reverse transcription at 53°C; 5 minutes initial denaturation at 94°C; 30 (tissue sample analysis) to 35 (cell lines) cycles of 20 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 30 seconds primer extension at 72°C; and 10 minutes at 68°C for the final extension.

The gene-specific primers for PAR-4 were as follows: sense 5'-CAC CGG AGG TGG TGA TGA ACA GCA -3' and antisense 5'-GAA GGT CTG CCG CTG CAG TGT CA -3'. The PCR conditions were identical to those described for PAR-3 except that 38 cycles of annealing were performed at 66°C.

The β-actin gene served as an internal control. The gene-specific primers for β-actin were as follows: sense 5'-CCA AGG CCA AAC CGC GAG AAG ATG-3' and antisense 5'-GGT ACA TGG TGC CGC CAG AC-3'. The PCR conditions were as follows: 30 minutes of reverse transcription at 55°C; 3 minutes of initial denaturation at 94°C; 20 cycles of 20 seconds denaturation at 94°C, 30 seconds of annealing at 66°C and 30 seconds of primer extension at 72°C; and 10 minutes at 68°C for the final extension.

The PCR products were fractionated on agarose gels containing 0.5µg/µl ethidium bromide. The experiments were documented using a computerised gel documentation system (Gel-Pro Analyzer™, Media Cybernetics, USA).

The RT-PCR products were quantified by size-fractionation by anion-exchange High-Performance Liquid Chromatography (HPLC) on a Waters LCM1 plus system (Waters Cooperation, Milford, MA, USA). The peak areas corresponding to the RT-PCR product at 260 nm were integrated and normalised to the level of the β-actin message (Millenium Software, Waters Cooperation, Milford, MA, USA).

Cell lysates for protein analysis

Whole-cell lysates of the six pancreatic cancer cell lines (MIA PaCa-2, PATU 8902, PATU 8988s, Capan-1, Capan-2 and DAN-G) and HUVECs were prepared by lysing the cells directly in RIPA buffer (660–900 µl). The surfaces of Falcon flasks were scrapped with a rubber policeman, and the lysate was removed to a microcentrifuge tube, homogenised via passage through a 21-gauge needle and incubated for 60 minutes on ice. The samples were then centrifuged at 10000Xg for 10 minutes at 4°C to remove debris. The protein content was measured (DC Protein Assay, Bio-Rad, Germany) and the lysates were stored at -80°C until use.

Western blot analysis

Western blot analysis was performed using 35 µg of protein in each lane. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For immunostaining, the membranes were incubated with the primary polyclonal anti-PAR-3 antibody (sc-5598; 1:1000; 4°C overnight) or the primary polyclonal anti-PAR-4 antibody (sc-1807; 1:1000; RT for 90 minutes), followed by incubation with the horseradish peroxidase-conjugated secondary antibody (sc-2371; 1:10,000; 45 minutes at RT). The immunoblotting results were visualised with a luminol-based chemiluminescence reagent and exposed to BIOMAX light-1 film (Kodak via Sigma Aldrich, Munich, Germany). For normalisation, each membrane was probed with an antibody against β-actin (sc-130657). The results were scanned and quantified.

Statistical analysis

All experiments were performed at least three times. Normalised PAR-3 RT-PCR products (µg/µl) and the results of the PAR-4 Western blot analysis (Integrated Optical Density, IOD) were used for statistical analysis with ANOVA in SPSS for Windows (IBM Germany, Ehningen, Germany). P-values ≥ 0.05 were considered statistically significant.

Results

Expression of PAR-3 mRNA

The expression of PAR-3 mRNA was determined in healthy pancreatic tissue and pancreatic adenocarcinoma samples. One pancreatic cancer cell line, PATU 8988s, was included in this experiment to compare the results from tissues and cell lines and rule out technical problems in RNA extraction from tissue samples.

High levels of PAR-3 were observed in healthy pancreatic tissue (1423 ± 669 µg/µl). However, in the corresponding pancreatic cancer tissue, the expression of PAR-3 (359 ± 13 µg/µl) was significantly

down regulated. The expression levels of PAR-3 were even lower in the pancreatic cancer cell line PATU 8988s ($23 \pm 3 \mu\text{g}/\mu\text{l}$) than in the pancreatic cancer tissue samples (Figure 1).

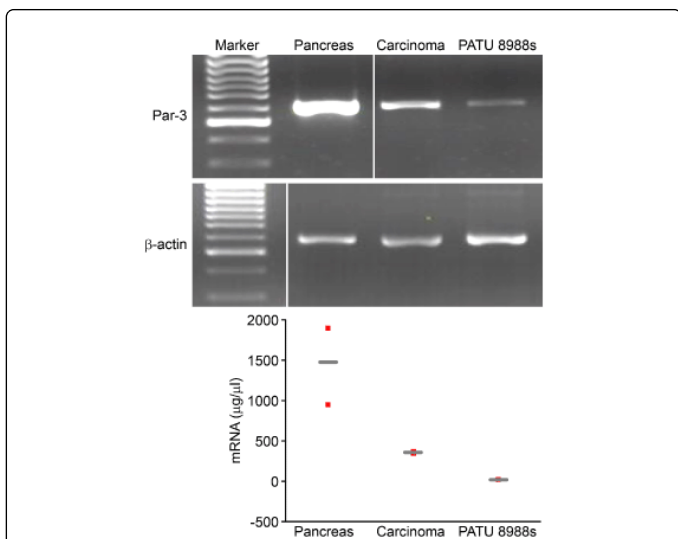


Figure 1: Expression of PAR-3 mRNA in tissue samples from a healthy pancreas (Lane 2), pancreatic cancer (Lane 3) and the pancreatic cancer cell line PATU 8988s (Lane 4), which was derived from a moderately differentiated pancreatic cancer specimen. The healthy pancreatic tissue showed strong PAR-3 expression. In pancreatic cancer tissue and in particular, the pancreatic cancer cell line, PAR-3 mRNA was significantly downregulated. The graph below shows the results of two independent experiments. The red dots represent the amounts measured by HPLC, and the grey bars indicate the mean.

The PAR-3 gene expression levels in the six pancreatic cancer cell lines with different grades of differentiation differed up to fivefold. The well-differentiated cell lines Capan-1 ($1250 \pm 145 \mu\text{g}/\mu\text{l}$) and Capan-2 ($1120 \pm 120 \mu\text{g}/\mu\text{l}$) exhibited comparatively high levels of PAR-3 expression. The moderately differentiated cell lines PATU 8902 ($785 \pm 5 \mu\text{g}/\mu\text{l}$), DAN-G ($756 \pm 66 \mu\text{g}/\mu\text{l}$), and PATU 8988s ($706 \pm 45 \mu\text{g}/\mu\text{l}$) exhibited markedly lower PAR-3 gene expression. The differences between the well-differentiated cell lines (Capan-1 and Capan-2) and the moderately differentiated cell lines (PATU 8902, PATU 8988s and DAN-G) were statistically significant ($p=0.005$ to $p<0.001$). Very low levels of PAR-3 expression were observed in MIA PaCa-2 ($232 \pm 11 \mu\text{g}/\mu\text{l}$), a cell line with poor differentiation and high malignant potential. This result was statistically significant compared to both Capan-1 and Capan-2, with $p<0.001$ (Figure 2).

PAR-3 protein expression

The protein expression of PAR-3 also correlated with the differentiation level of the pancreatic cancer cell lines. In the well-differentiated cell lines Capan-1 and Capan-2 and in DAN-G, PAR-3 protein expression was observed, but no difference in expression between these cell lines was observed.

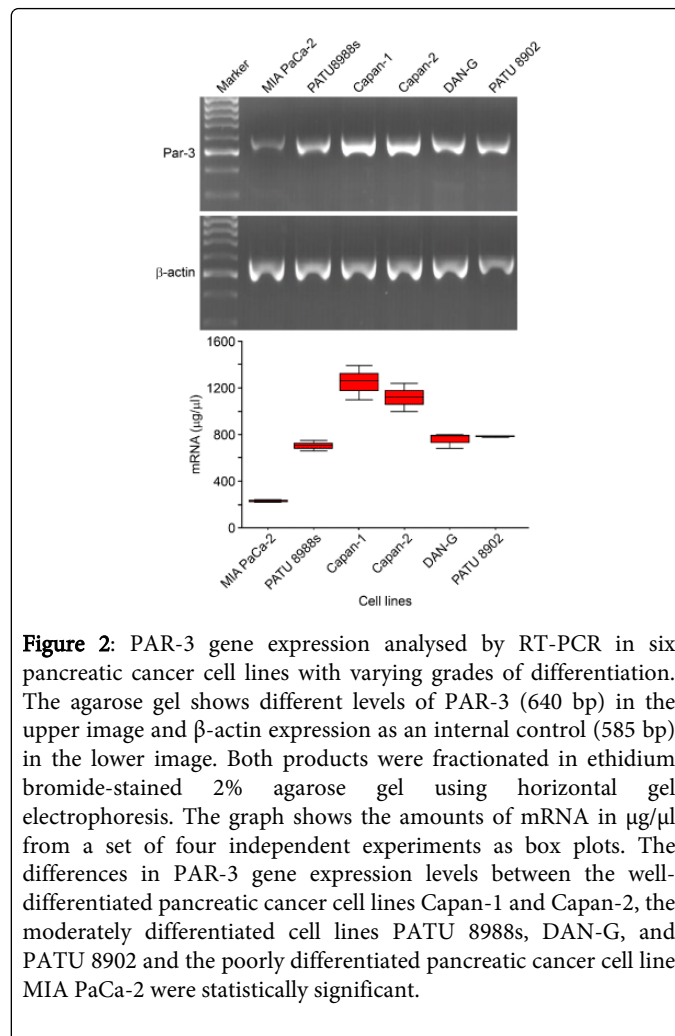


Figure 2: PAR-3 gene expression analysed by RT-PCR in six pancreatic cancer cell lines with varying grades of differentiation. The agarose gel shows different levels of PAR-3 (640 bp) in the upper image and β -actin expression as an internal control (585 bp) in the lower image. Both products were fractionated in ethidium bromide-stained 2% agarose gel using horizontal gel electrophoresis. The graph shows the amounts of mRNA in $\mu\text{g}/\mu\text{l}$ from a set of four independent experiments as box plots. The differences in PAR-3 gene expression levels between the well-differentiated pancreatic cancer cell lines Capan-1 and Capan-2, the moderately differentiated cell lines PATU 8988s, DAN-G, and PATU 8902 and the poorly differentiated pancreatic cancer cell line MIA PaCa-2 were statistically significant.

No PAR-3 protein expression was detected in the moderately differentiated cell lines PATU 8988s and PATU 8902 and the poorly differentiated cell line MIA PaCa-2 (Figure 3).

Expression of PAR-4 mRNA

High mRNA expression levels of the thrombin receptor PAR-4 were observed in healthy pancreatic tissue. However, PAR-4 mRNA was downregulated in the corresponding pancreatic cancer tissue to below the detectable level (Figure 4). Accordingly, PAR-4 gene expression was below the detectable level in all RNA samples from the tested pancreatic adenocarcinoma cell lines. Consequently, no statistical analysis could be performed. As a control, PAR-4 mRNA was detected in endothelial cells (HUVECs), ruling out primer dysfunction or technical problems (Figure 5).

PAR-4 protein expression

Weak PAR-4 protein expression was detected in all pancreatic adenocarcinoma cell lines tested. The expression levels of PAR-4 did not differ between the different cell lines. In the endothelial cells (HUVECs) used as a control, a strong PAR-4 protein signal was observed (Figure 6).

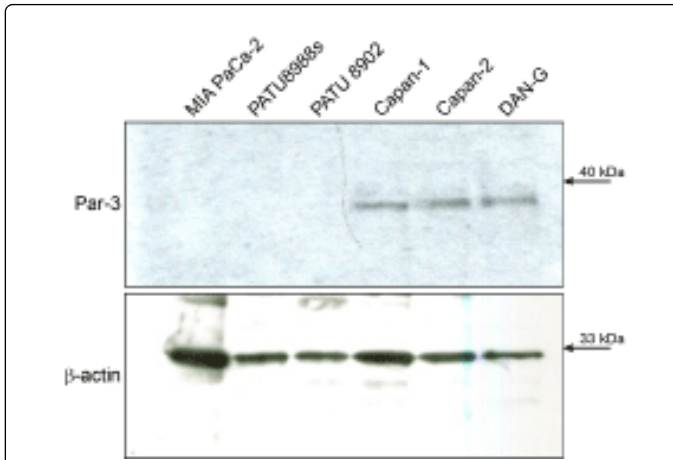


Figure 3: Representative Western blot analysis of PAR-3 protein expression in whole cell lysates of six different pancreatic cancer cell lines (MIA PaCa-2, PATU 8988s, PATU 8902, Capan-1, Capan-2 and DAN-G). The upper image shows that PAR-3 is only expressed in the pancreatic cancer cell lines Capan-1, Capan-2, and DAN-G. The PAR-3 protein has a size of approximately 40 kDa. The lower image shows the membrane reprobbed for β -actin, which was used as an internal control, at 33 kDa. Although the β -actin expression varies between the cell lines, all cell lines show expression, thereby ruling out technical faults.

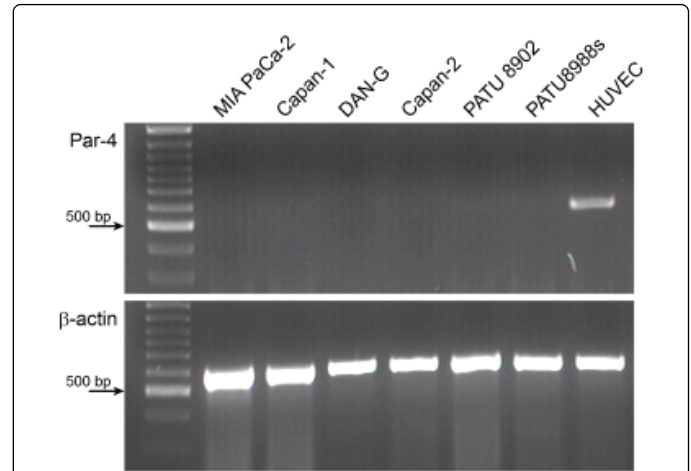


Figure 5: Representative RT-PCR analysis of PAR-4 expression using total RNA from six different pancreatic cancer cell lines (Lanes 2-7) and HUVECs (Lane 8). The upper image shows the PAR-4 PCR product, which has a size of 581 bp. The lower image shows the PCR products for β -actin, a housekeeping gene that served as an internal control, at 598 bp. Lane 1 in both pictures shows the molecular weight marker (100 bp PLUS, PeqLab, Erlangen, Germany); the band corresponding to 500 bp is indicated by the arrow.

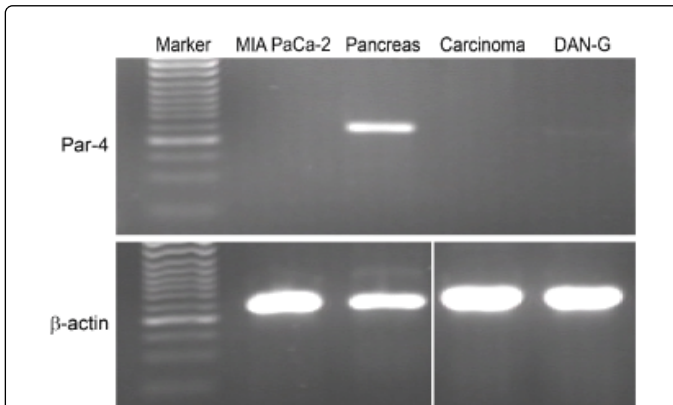


Figure 4: Representative RT-PCR analysis of the expression of PAR-4 mRNA in tissue samples from a normal pancreas (Lane 3), pancreatic cancer (Lane 4) and the two pancreatic cancer cell lines MIA PaCa-2 (Lane 2) and DAN-G (Lane 5). Strong PAR-4 expression was observed in healthy pancreatic tissue. PAR-4 mRNA could not be amplified from the pancreatic cancer tissue and the pancreatic adenocarcinoma cell lines.

Discussion

Patients with pancreatic carcinoma frequently suffer from thrombosis as a result of an excessively activated coagulation cascade and thrombin generation [6,9,10,36]. The thrombin receptor PAR-1 plays a role in the activation of platelets and is thought to be the link between thrombin and the activation of oncopathogenic potential in both benign and malignant cells [22-24]. The effects of thrombin on pancreatic cancer progression and the associated underlying mechanisms by which the diverse thrombin receptors, particularly PARs, are orchestrated are still not completely understood.

In this study, we investigated the expression of the recently cloned receptors PAR-3 and PAR-4 in pancreatic cancer tissue and cell lines for the first time.

Gene expression of PAR-3 was detected in healthy pancreatic tissue. In contrast with its high expression in healthy tissue, the level of PAR-3 mRNA was significantly decreased in pancreatic cancer tissue and pancreatic cancer cell lines.

Furthermore, the level of PAR-3 gene expression in six pancreatic cancer cell lines correlated with the degree of differentiation of the cell lines. PAR-3 gene expression was lower in the moderately differentiated pancreatic cancer cell lines (PATU 8988s, DAN-G, and PATU 8902) compared with the well-differentiated pancreatic cancer cell lines (Capan-1 and Capan-2). The downregulation of PAR-3 mRNA expression was most evident in the poorly differentiated pancreatic cancer cell line MIA PaCa-2. PAR-3 expression was up to fivefold lower in MIA PaCa-2 than in the better differentiated pancreatic cancer cell lines.

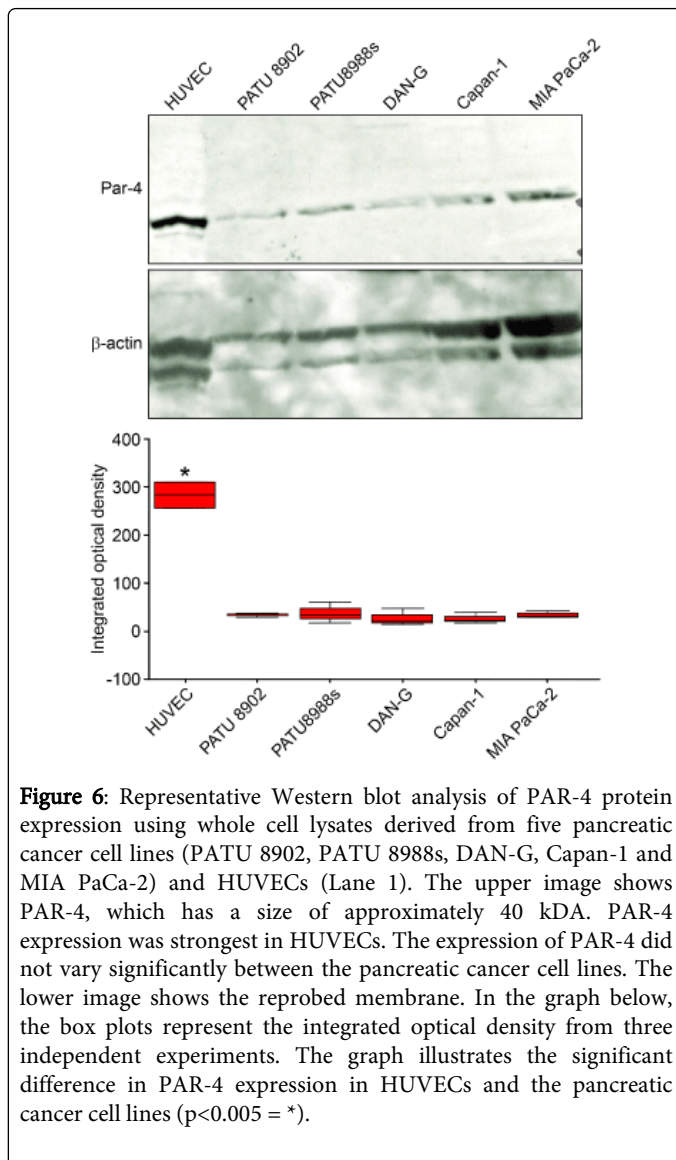


Figure 6: Representative Western blot analysis of PAR-4 protein expression using whole cell lysates derived from five pancreatic cancer cell lines (PATU 8902, PATU 8988s, DAN-G, Capan-1 and MIA PaCa-2) and HUVECs (Lane 1). The upper image shows PAR-4, which has a size of approximately 40 kDA. PAR-4 expression was strongest in HUVECs. The expression of PAR-4 did not vary significantly between the pancreatic cancer cell lines. The lower image shows the reprobbed membrane. In the graph below, the box plots represent the integrated optical density from three independent experiments. The graph illustrates the significant difference in PAR-4 expression in HUVECs and the pancreatic cancer cell lines ($p < 0.005 = *$).

The analysis of PAR-3 protein expression in the pancreatic cancer cell lines revealed specific variations. As expected, protein expression was detected in the well-differentiated cell lines Capan-1 and Capan-2. However, similar protein expression was confirmed for the moderately differentiated cell line DAN-G, whereas none of the other cell lines exhibited protein expression of PAR-3.

The mechanism of PAR-3 activation remains unknown. PAR-3 was first described in PAR-1-deficient mice, the platelets of which are fully responsive to thrombin [37]. Although the PAR-3 consensus sequence contains a putative thrombin cleavage site, no synthetic peptide is able to mimic receptor activation and function [38]. Interestingly, the tethered ligand peptide for PAR-3 interacts with PAR-1 and PAR-2 in Jurkat cells [39]. In mice, PAR-3 functions as a co-factor for PAR-4 in platelet activation [40].

In human endothelial cells, PAR-3 regulates PAR-1 signalling by receptor dimerisation, resulting in increased endothelial permeability [41]. In human rhabdomyosarcoma, PAR-3 inhibits tumor cell proliferation and growth [42].

Because PAR-3 is an apparent factor in the inhibition of tumor proliferation, the observed downregulation of PAR-3 emphasises the PAR-3's putative role in controlling tumor growth and malignant potential. Because of receptor heterodimerisation, the downregulation of PAR-3 further increases the oncogenic potential of PAR-1 in pancreatic cancer.

The mRNA of the thrombin receptor PAR-4 was expressed in healthy pancreatic tissue. In the corresponding tissue from pancreatic cancer, no PAR-4 mRNA was detectable. Accordingly, PAR-4 mRNA expression was below detectable levels in all of the pancreatic cancer cell lines.

Contrary to the gene expression results, PAR-4 protein expression was detectable in all whole-cell lysates of the pancreatic cancer cell lines, although the detection levels were low. Post-transcriptional and post-translational modifications, a well-known phenomenon for G protein-coupled receptors, may explain the discrepancy between the mRNA and protein results in our experiments [43].

Both PAR-4 and PAR-1 are activated by thrombin via the cleavage of a specific extracellular domain. Receptor activation and function can be mimicked by synthetic peptides representing the tethered ligand sequence. Interestingly, PAR-4 requires much higher concentrations of thrombin (1 U/ml or greater) than PAR-1 for activation and might serve as a modulator of the cellular dose-response to thrombin [28]. Thus, it has been hypothesised that PAR-4 might induce conflicting effects if it serves as a natural shut-off for PAR-1. This relationship has been confirmed for the effect of PAR-1 (contraction) and PAR-4 (relaxation) on the muscularis mucosae in the rat oesophagus [44].

If PAR-4 also serves as a shut-off for PAR-1 at high thrombin concentrations in a cancer setting, our results indicate that the downregulation of PAR-4 together with the upregulation of PAR-1 in pancreatic cancer strengthens the malignant potential of thrombin because the regulatory function of PAR-4 is missing.

Our results are supported by the reported PAR-4 expression in human lung adenocarcinoma. PAR-4 expression is decreased in lung adenocarcinoma and is associated with poor differentiation and metastasis [45].

In a murine model using a melanoma cell line, PAR 4 $-/-$ mice displayed a significantly lower tumor burden and less distant metastasis [21], but this model does not address the role of PARs in the tumor cells themselves.

Our results reveal a contradictory regulatory mechanism for the expression of the thrombin receptors PAR-3 and PAR-4 compared with PAR-1 in pancreatic cancer tissue and pancreatic adenocarcinoma cell lines. In contrast to PAR-3 and PAR-4, the thrombin receptor PAR-1 is not expressed in healthy pancreatic tissue. However, PAR-1 is upregulated during malignant transformation, and the level of PAR-1 expression correlates with the grade of malignancy [25]. By contrast, PAR-3 and PAR-4 are downregulated during tumorigenesis and the de-differentiation of pancreatic cancer.

The downregulation of both PAR-3 and PAR-4 in pancreatic cancer compared with healthy pancreatic tissue further supports a role of both receptors in cancer progression.

Furthermore, if PAR-4 act as a shut-off for PAR-1 and PAR-3 also serves as an important receptor for heterodimerisation in PAR-1 signalling in the pancreas, our experiments would imply that

pancreatic cancer progression is governed by both the upregulation of PAR-1 and the downregulation of PAR-3 and PAR-4.

Conclusion

We demonstrated that the thrombin receptors PAR-3 and PAR-4 are both present in healthy pancreatic tissue. Our results show that both receptors are significantly downregulated in pancreatic cancer tissue and pancreatic cancer cell lines. The regulation of PAR-3 and PAR-4 contrasts with that of the thrombin receptor PAR-1, which is merely detectable in the healthy pancreas but is significantly upregulated in pancreatic cancer tissue and pancreatic cancer cell lines.

Although little is known about the role of PAR-3 and PAR-4 in pathophysiological mechanisms, our results imply their involvement in the progression of pancreatic cancer. Given the key role of thrombin in the context of tumor progression, the role of the thrombin receptors PAR-3 and PAR-4 in pancreatic cancer merits further study.

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