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ORIGINAL ARTICLE

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Expression of functional protease-activated receptor 1 in human prostate cancer cell lines

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Abstract Functional protease-activated receptors (PAR) are expressed by a variety of malignant cells. In the present study, RT-PCR assays demonstrated the expression of the thrombin receptor PAR-1 mRNA in human prostate cancer cell lines DU 145, LnCAP, and SV40-immortalized human prostate epithelial cell line PNT1A. In contrast, the additional thrombin receptors PAR-3 and PAR-4 were not detected. PAR-1 protein localized to the cellular surface was detected by flow cytometry in all three cell lines. To demonstrate the functional importance of the PAR-1, the effects of different concentrations of thrombin on cell proliferation kinetics were assessed. The treatment of growth-arrested cells with varying concentrations of thrombin demonstrated dose- and time-dependent effects. At low concentration (<0.5 U/ml), thrombin induced proliferation of all prostate-derived cell lines. Thrombin at higher concentration (1.0 U/ml) initially stimulated PNT1A and LnCAP cells to proliferate (time of thrombin application 24 h and 48 h) followed by inhibited growth when assessed after 72 h of incubation. In contrast, 1.0 U/ml thrombin caused earlier inhibition of DU 145 proliferation starting at 48 h of incubation. Our results suggest that PAR-1 mediates the proliferation-modulating effects of thrombin on prostate cancer cells.

Keywords Protease-activated receptor · Thrombin · Prostate cancer · Proliferation

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Introduction

In addition to its central role in blood coagulation, thrombin elicits a number of cellular effects by proteolytically activating specific thrombin receptors. Following the cloning of the thrombin receptor [24, 29], a family of related seven transmembrane domain-containing G protein-coupled protease-activated receptors (PARs) consisting of four members has emerged [4], of which PAR-1, PAR-3 and PAR-4 are activated by thrombin. Physiologically, the prototypic PAR-1 is expressed by different tissues including blood and blood vessel cells [8], and the central nervous system [32]. In addition, PAR-1 has been detected in malignant cells from different locations [12, 19, 25, 33].

Thrombin has a variety of in vitro effects on cancer cells, including modulation of their proliferation, adhesion, and invasiveness [1, 15, 18, 30], with most of these effects being attributable to the activation of thrombin receptors. Moreover, animal studies have provided evidence that PAR-1 is critically involved in tumor growth and metastasis [6, 20].

Circumstantial evidence predicts the expression of thrombin receptors by prostate cancer cells. Thrombin treatment of prostate cancer cell lines variably triggers elevated intracellular free calcium signals depending on the cell lines used [16, 31]. In addition, thrombin induces the synthesis and secretion of vascular endothelial growth factor in DU 145 prostate cancer cells [10]. A bimodal effect of thrombin on the proliferation rate of DU 145 cells has been reported with enhanced growth at low concentration and impaired growth/apoptosis at higher concentrations [36].

In the present study, we attempted to demonstrate the expression of protease-activated receptors by several established prostate-derived cell lines. In addition, the function of PAR-1 in these cells was characterized by the thrombin-induced modulation of cell proliferation.

Materials and methods

Cell culture

Human prostate cancer cell lines DU 145 [28] and LnCAP [7] were obtained from the American Type Culture Collection (Manassas, Va., USA). SV 40-immortalized human prostatic epithelial cells designated PNT1A [5] were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2% glutamine, and penicillin/streptomycin (Biochrom, Berlin, Germany) with subculturing performed at a split ratio of 1:3. Subcultured cells were allowed to recover for 2 days in complete medium before performing experiments.

RNA isolation and RT-PCR

Total RNA was extracted from subconfluent cells by the guanidine isothiocyanate procedure using the TRIzol reagent (Life Technologies, Karlsruhe, Germany) and digested with 1 unit DNase per µg RNA. Total RNA (2 μg) was reverse transcribed using random hexamers and the Superscript II preamplification system (Life Technologies).

Amplifications were performed using a Personal Cycler (Biometra, Göttingen, Germany) in 50 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 mM each desoxynucleotide triphosphate, 0.2 µM of each primer, and 2 units of Taq DNA polymerase (QIAGEN, Hilden, Germany). After an initial denaturation step (5 min at 94°C), 30 thermal cycles were performed (1 min denaturation at 94°C, 1 min annealing as indicated, 1 min synthesis at 72°C), followed by a 7 min final extension step.

The following primers were used: PAR-1 forward 5′-gtg ctg ttt gtg tct gtg ct and reverse 5′-cct ctg tgg tgg aag tgt ga [33], annealing temperature 55°C, product size 598 bp; PAR-3 forward 5′-tcc cct ttt ctg cct tgg aag and reverse 5′-aaa ctg ttg ccc aca cca gtc cac [27], annealing temperature 55°C, product size 513 bp; and PAR-4 forward 5′-ggt gcc cgc cct cta tgg and reverse 5′-tcg cga ggt tca tca gca [36], annealing temperature 60°C, product size 121 bp.

PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Flow cytometry

Flow cytometry was performed according to established procedures [17]. In brief, subconfluent cell monolayers were dissociated by exposure to 50 mM EDTA, washed and a single cell suspension prepared in PBS containing 2% bovine serum albumin (PBS-BSA). A total of 5×10^5 cells were incubated with 5 µg mouse monoclonal anti-human PAR-1 IgG1 (clone WEDE15, Beckman Coulter Immunotech, Krefeld, Germany) recognizing both uncleaved and cleaved PAR-1 species (4°C, 30 min). After subsequent washing, phycoerythrin-conjugated goat $F(ab')_2$ anti-mouse IgG was added for 30 min. The cells were washed twice in PBS-BSA and 10,000 cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Isotypic murine monoclonal antibody (clone MsIgG1) was used as negative control.

Proliferation assay

Cells were seeded at an initial density of 5,000 cells/100 µl/well in sterile 96-well microtitre plates (Sarstedt, Nümbrecht, Germany). After overnight attachment in complete cell culture medium, cells were growth-arrested using RPMI 1640 containing 0.1% BSA for 48 h. Human thrombin (Enzyme Research Laboratories, South Bend, Ind., USA) was diluted in the same medium and applied for the time periods as indicated. To verify that the effects exerted by thrombin are dependent on its proteolytic activity, control experiments were performed in which the irreversible thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK, Calbiochem, Bad Soden, Germany) was added to thrombin-containing culture media (10 µg/ml) for 30 min before application to the cells. Using the thrombin-specific chromogenic substrate S-2238 (Haemochrom, Essen, Germany), it was confirmed that no residual thrombin activity remained after PPACK pretreatment [14]. PPACK itself had no detectable effect on cell proliferation (data not shown). Cell proliferation was assessed by a commercially available colorimeric assay based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase activity (WST-1, Roche, Mannheim, Germany). A total of 10 µl WST-1 reagent was added to each well, incubated for 30 min at 37°C and the absorbance read at 450 nm using a microtitre plate reader (400 SF, SLT Labinstruments, Salzburg, Austria). Background absorbance was controlled in separate wells by adding WST-1 to 100 µl RPMI 1640 containing 0.1% BSA. Preliminary experiments demonstrated a linear relationship between absorbance and cell numbers over the range covered by the experiments (data not shown). The proliferation index was calculated as the percentage of cell number in stimulated versus control cell cultures.

Statistics

Results are presented as means ± SEM. Differences between group means were analysed by Student's two tailed *t*-test. Differences were considered significant at $P \leq 0.05$.

Results

Detection of mRNA and protein for PAR-1

Using primers specific for PAR-1, a single 598 bp product was amplified by RT-PCR from total RNA obtained from two prostate cancer cell lines (LnCAP, DU 145) and SV 40-immortalized prostatic epithelial cells (PNT1A). Negative controls included the substitution of cDNA with DNase-digested total RNA (1 µg) or nuclease-free sterile water and yielded no amplification product (Fig. 1A). Further RT-PCR experiments demonstrated that the thrombin receptors PAR-3 and PAR-4 were not expressed by the prostate-derived cell lines under investigation (Fig. 1B, C). The feasibility of the experimental approach was verified by including cDNA synthesized from total RNA obtained from human umbilical vein endothelial cells (HUVEC) as positive controls.

Employing flow cytometry, PAR-1 protein located at the cellular surface was detected in all three prostate-derived cell lines investigated (Fig. 2).

Proliferation assay

To demonstrate that PAR-1 acts as a functional thrombin receptor, different concentrations of thrombin were applied to growth-arrested cells and their effects on cell proliferation estimated after 24, 48, and 72 h. Low concentrations of thrombin caused an increase in cell proliferation in DU 145 cells at all points

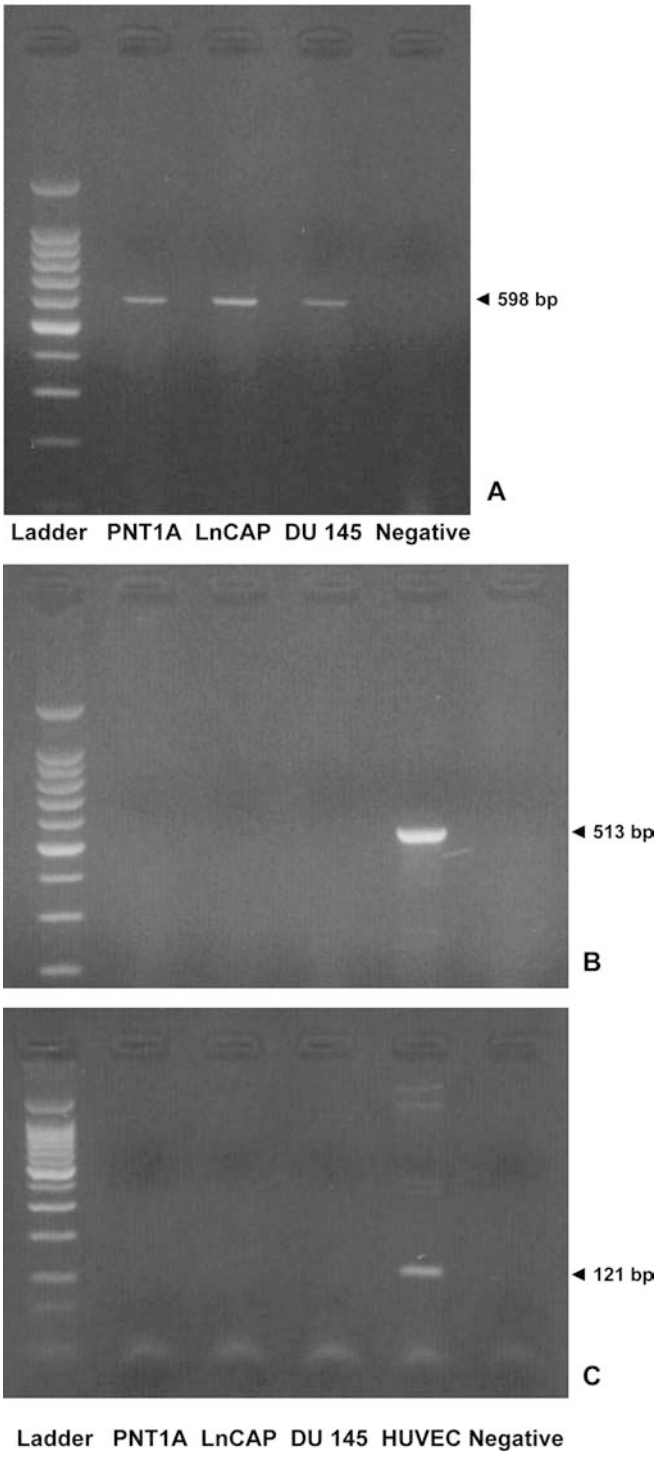

Fig. 1 Demonstration of protease-activated receptors (PAR) mRNA by RT-PCR in cultured prostate-derived cells. Total cellular RNA was reverse-transcribed and cDNAs amplified by PCR using specific primers. Ethidium bromide-stained gels are shown. Amplifications yield single PCR product of the predicted length (**A** PAR-1: 598 bp, ; **B** PAR-3: 51 bp; **C** PAR-4: 121 bp). *HUVEC* denotes human umbilical vein endothelial cells as positive controls for PAR-3 and PAR-4

of time determined (0.1 U/ml: $P=0.03$ for 48 vs 24 h, $P<0.0001$ for 72 vs 48 h; 0.5 U/ml: $P=0.0001$ for 48 vs 24 h, $P=0.04$ for 72 vs 48 h). In contrast, 1.0 U/ml thrombin induced an increased cell proliferation at 24 h followed by a steady decrease ($P=0.07$ for 48 vs 24 h, $P<0.0001$ for 72 vs 48 h) to reach cell numbers similar to control cells left in RPI 1640 medium containing 0.1% BSA (Fig. 3A). PNT1A cells demonstrated significantly increased cell numbers for thrombin applied at 0.1 U/ml which was almost linear over time ($P=0.005$ for 48 vs 24 h, $P=0.02$ for 72 vs 48 h). Thrombin at 0.5 U/ml caused the initially increased proliferation rate ($P=0.04$ for 48 vs 24 h) to reach a plateau between 48 and 72 h ($P=0.82$). A higher thrombin concentration (1.0 U/ml) caused increased cell proliferation after 24 h which continued to rise after 48 h without reaching significance ($P=0.20$) followed by a sharp decrease between 48 and 72 h ($P=0.002$). Similarly to DU 145 cells, 1.0 U/ml thrombin caused PNT1A cell numbers equivalent to untreated control cells after 72 h (Fig. 3B). LnCAP cells demonstrated a different proliferation response to thrombin treatment. After 24 h, no differences were observed for thrombin-treated cells at any thrombin concentration compared to control cells. All thrombin concentrations induced a significant increase in cell numbers after 48 h (0.1 U/ml: $P=0.0001$; 0.5 U/ml: $P=0.006$; 1.0 U/ml: $P=0.007$). Cells treated with 0.1 U/ml thrombin reached a plateau ($P=0.67$ for 72 vs 48 h). Thrombin at 0.5 U/ml caused a slight decrease in cell numbers ($P=0.38$) whereas cell numbers after thrombin treatment with 1 U/ml returned to control values ($P=0.003$; Fig. 3C).

The irreversible thrombin inhibitor PPACK was used to block thrombin's proteolytic activity. Preincubation of 1.0 U/ml thrombin with 10 μg/ml PPACK for 30 min before the addition of thrombin completely abolished thrombin-induced changes in cellular proliferation (Fig. 4) demonstrating that thrombin's proteolytic activity is essential for its potential to modulate cell proliferation.

Discussion

In the present study, we demonstrated the expression of PAR-1 at the mRNA and protein level by two different prostate cancer-derived cell lines and SV 40-immortalized prostatic epithelial cells, thus providing evidence that the prototypic functional protease-activated receptor is synthesized and localized at the surface of these cells in culture. Although no attempts were undertaken to quantitate PAR-1 mRNA in the present investigation, it should be emphasized that a single-round RT-PCR approach yielded amplification products. In contrast, using identical primers for PAR-1, another study [33] did not obtain unequivocal evidence for the presence of PAR-1 mRNA in malignant cells

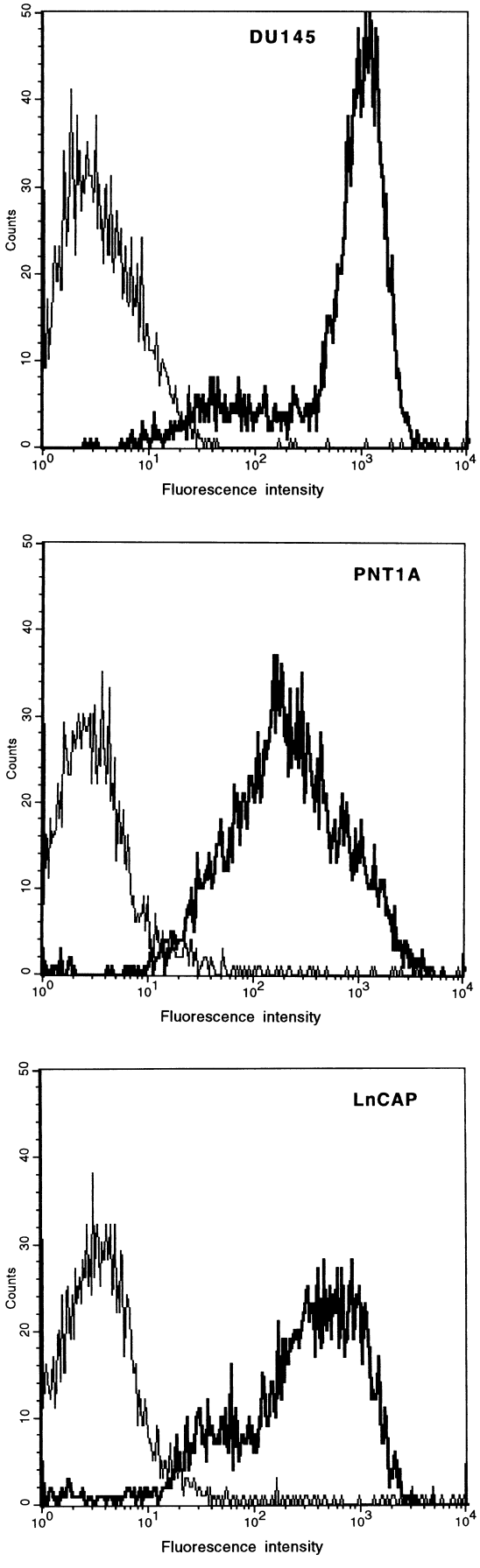

Fig. 2 Flow cytometric detection of PAR-1 in prostate-derived cell lines using a monoclonal antibody recognizing both uncleaved and cleaved PAR-1 species: logarithmic fluorescence intensity of PAR-1 staining (*bold line*) versus isotypic control (*fine line*).

making a nested amplification protocol necessary to clearly demonstrate amplification products.

It has recently been reported that human astrocytoma cells express a dual thrombin receptor system consisting of PAR-1 and PAR-4 [13]. Similarly, a complex PAR expression profile was reported in breast cancer cells [11]. The present study provides evidence that three prostate-derived cell lines exclusively express the thrombin receptor PAR-1 thus extending the observation of PAR-1 expression in DU 145 cells [10] to additional cell lines widely used as cellular models of prostatic disease. However, it remains to be investigated if other thrombin-binding molecules, including glycoprotein Ib (GPIb)-related receptors and thrombomodulin, are expressed by prostate-derived cells as has been variably demonstrated for malignancies of different origin [21, 22]. Although a likely scenario, it seems premature to conclude that cellular effects evoked by the exposure of malignant prostate cells to thrombin are exclusively mediated by the proteolytic activation of its prototypic functional receptor PAR-1.

It has recently been shown that thrombin causes concentration-dependent dual effects on tumor cells with lower thrombin concentrations (<0.5 U/ml) leading to increased cell proliferation, while thrombin concentrations exceeding 0.5 U/ml induced impaired tumor cell growth [36]. Suppressed tumor cell growth was associated with cell cycle arrest and the induction of apoptosis. Notably, only one representative prostate cancer-derived cell line (DU 145) was included in the study. We present data on three different prostate-derived cell lines and provide evidence that the cell growth response to varying thrombin concentrations critically depends on the cell line under consideration. Nevertheless, although kinetically different, all cell lines used in the present investigation reacted with disturbed cell growth upon treatment with a higher thrombin concentration (1.0 U/ml) for 2–3 days. Interestingly, the highest thrombin concentration selected never caused cell numbers below control values, i.e. growth-arrested cells maintained in RPMI 1640 supplemented with 0.1% BSA.

Potential mechanisms underlying the different cellular proliferation kinetics in the cell lines used include the impact of thrombin on cell cycle control and apoptosis. Thrombin-induced cell cycle control and apoptosis has been associated with p53-independent, STAT-1-dependent upregulation of $p21^{waf/cip1}$ and caspases [9]. However, inconsistent patterns of induction and/or modulation of p53, p21 and bcl-2 in thrombin-induced apoptosis have been described [1]. Preliminary evidence suggests the involvement of a variety of signaling mechanisms in the induction of apoptosis by thrombin in malignant cell lines [2]. Considering the fact that G

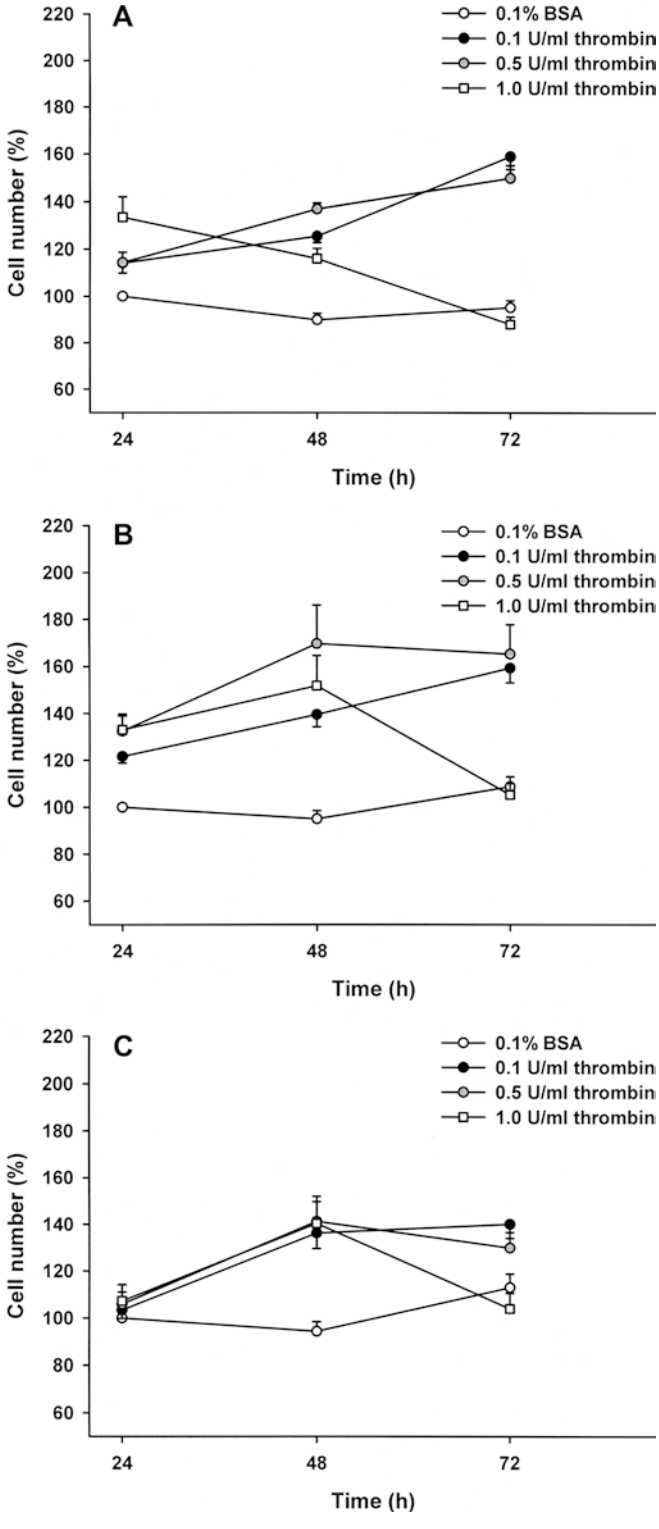

Fig. 3 Cell proliferation studies using: **A** prostate-derived cells DU 145 , **B** PNT1A, and **C** LnCAP. Growth-arrested cells were treated with different thrombin concentrations and compared to control cells kept in RPMI 1640 supplemented with 0.1% BSA. Cell numbers were estimated at the time points indicated using a microtitre plate-adapted version of the mitochondrial dehydrogenase activity assay (WST-1, Roche, Mannheim, Germany). Three independent experiments with from four to six replicates of each treatment were performed with each cell line

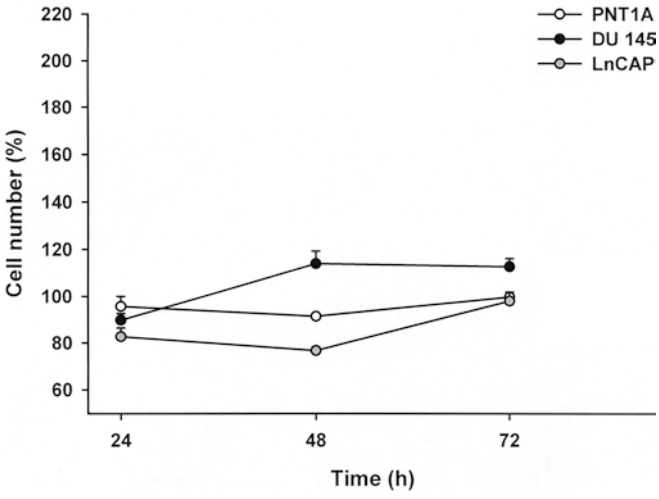

Fig. 4 Cell proliferation analysis using thrombin (1.0 U/ml) after pretreatment with PPACK (10 mg/ml)

protein-coupled receptors may lead to activated ectoproteases [23], subsequent generation of RGD peptides with known apoptosis-inducing activity [3] could be another mechanism of thrombin-induced apoptosis in malignant cells. The molecular mechanisms underlying the different cell growth response to higher thrombin concentrations obtained in the present study require additional investigation.

The opposite effects of thrombin have been described in rat C6 glioma cells, with inhibition of proliferation at lower concentrations of thrombin (<0.1 U/ml) and stimulation of proliferation when applied at concentrations exceeding 0.1 U/ml [26]. Although the molecular mechanisms have not been elucidated, it is tempting to speculate that the proliferation-modulating cellular effects of thrombin depend on a multitude of factors inherent to the cell line under consideration, including the PAR expression profile, presence of additional cellular thrombin-binding sites, thrombin activity-neutralizing capacity to suggest but a few.

The modulation of tumor cell proliferation by thrombin requires the presence of enzyme activity in malignant tissue. Immunohistochemical analysis demonstrated the presence of prothrombin fragment $1+2$ in cancer tissues indicating local thrombin generation [34]. Using hirudin as a specific label, staining for enzymatically active thrombin was reported for tumor cells of different tissue origin [35]. Although it seems conceivable to propose the presence of enzymatically active thrombin in malignant tissue, estimates of its intratumoral activity are not available at present.

In conclusion, our results demonstrate that functionally active PAR-1 is expressed by different prostate-derived cell lines. Its proteolytic activation by thrombin dose-dependently changes cell proliferation rates with lower thrombin concentrations causing increased cell proliferation and higher concentrations leading to cell cycle arrest/apoptosis. Further studies are warranted to investigate the importance of throm-

bin-induced modulation of cell proliferation rates in human prostate cancer as well as to delineate the molecular mechanisms underlying the observed bimodal effect of different thrombin concentrations on prostate cell proliferation.

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