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Thrombin/thrombin receptor (PAR-1)-mediated induction of IL-8 and VEGF expression in prostate cancer cells

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Abstract

Interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF) are two cytokines promoting prostate tumor growth and angiogenesis. The main coagulation protease thrombin may modulate the malignant phenotype of prostate cancer cells via its cellular receptor(s). We aimed to investigate the effects of thrombin on IL-8 and VEGF expression in DU 145 prostate cancer cells. Thrombin induced the expression and secretion of IL-8 and VEGF, with more pronounced effects on IL-8. Target-specific siRNA-induced protease-activated receptor 1 (PAR-1) knockdown completely neutralized thrombin-enhanced cytokine secretion, demonstrating the essential role of PAR-1. Inhibitors of either extracellular signal-regulating kinase (ERK) or phosphatidylinositol 3-kinase (PI3K) partly reversed the thrombin-induced cytokine expression, suggesting that both ERK and PI3K kinase pathways may be involved in IL-8 and VEGF expression. The results suggest that the thrombin/PAR-1 system upregulates cytokines in prostate cancer cells which in turn may contribute to the progression of prostate cancer.
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Although the detailed role of blood coagulation factors in the pathogenesis of a variety of malignancies is still incompletely understood, abnormal plasma coagulation profiles were repeatedly reported in prostate cancer patients [1]. A major focus of ongoing clinical and experimental research is to provide compelling evidence for the local activation of the coagulation system including its central enzyme thrombin, which may in turn contribute to the development and progression of cancer [2,3]. In the past, only limited aspects of the functional importance of thrombin as related to prostate cancer biology have been elucidated [4]. Using prostate cancer cell lines, it has been demonstrated that thrombin may modulate cell proliferation, adhesion to extracellular matrix components, and the secretion of the proangiogenic vascular endothelial growth factor (VEGF) [5–7].

Accumulating evidence indicates that thrombin exerts its cellular functions through proteolytic activation of its G protein-coupled transmembrane receptors referred to as protease-activated receptor (PAR) 1, 3, and 4 [8]. Although PAR-1 seems to mediate most effects of thrombin, the complexity of thrombin-mediated receptor activation has only been recently recognized. The presence and functionality of multiple thrombin-activated PAR species have been observed in different malignancies [9–12]. Furthermore, PAR-independent thrombin effects on cell proliferation and apoptosis have been reported [13–16]. The available data suggest that the molecular mechanisms of thrombin critically depend on the cell type and its thrombin receptor repertoire. Gene silencing at the post-transcriptional level by RNA interference (RNAi) may represent a potential tool to gain further insight into the thrombin/thrombin receptor interface in cancer.

Data from in vitro and in vivo experiments suggest that interleukin 8 (IL-8) and VEGF are two multifunctional cytokines implicated in malignant growth. IL-8 is a potent

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inducer of tumor growth, migration, invasion, and angiogenesis [17–19], while VEGF is a proangiogenic factor promoting tumor progression [20]. Circumstantial evidence predicted that thrombin upregulates IL-8 production from non-malignant cell types [16,21]. However, thrombin's effects on IL-8 expression and its molecular basis in tumor cells remain controversial [18,22]. One previous report demonstrated that thrombin induces VEGF expression in FS4 fibroblasts, CHRF megakaryocytes, and DU 145 prostate cancer cells, and provided evidence of kinase signaling mechanisms involved in thrombin-induced VEGF production in FS4 fibroblasts [7].

The present study aimed to investigate the effects of thrombin on the expression of IL-8 and VEGF from DU 145 prostate cancer cells. The mediating role of PAR-1 was examined by gene silencing using target sequence-specific small interfering RNAs (siRNAs). In addition, kinase inhibitors were used to study the putative involvement of two major kinase signaling pathways, extracellular signal-regulating kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), in thrombin/PAR-1-induced cytokine expression.

Materials and methods

Cell culture and treatment. DU 145 human prostate cancer cells (ATCC, Manassas, VA, USA) were routinely kept in RPMI 1640 medium supplemented with 10% fetal calf serum, 2% glutamine, and penicillin/streptomycin (complete medium). For thrombin treatment, 2×10^5 cells were seeded in each well of 12-well tissue culture plates with complete medium. After 30 h of incubation, confluent cells were growth-arrested overnight with serum-free medium. The cells were treated with different concentrations of thrombin (Enzyme Research Laboratories, South Bend, IN, USA). After time intervals as indicated, the conditioned cell culture supernatants were harvested, centrifuged, and stored at −80 °C for determination of cytokine concentrations. The cell monolayers were extracted using TRIzol reagent (Life Technologies, Karlsruhe, Germany) for mRNA analysis (see below). In part of the experiments, thrombin was pretreated with its irreversible inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK, Calbiochem, Bad Soden, Germany) at 10 μg/ml final concentration. To investigate the kinase signaling pathways involved in thrombin-modulated cytokine secretion, either the specific ERK inhibitor PD98059 (20 μM, Calbiochem) or the specific PI3K inhibitor LY294002 (25 μM, Sigma, St. Louis, MO, USA) was applied to quiescent cells for 30 min before the addition of thrombin in the presence of the kinase inhibitor.

Design of small interfering RNAs against PAR-1. An online tool (Qiagen, Hilden, Germany) was used for searching potential siRNA targets with a length of 21 bases in the human PAR-1 gene (gi 6031164). Following comparison of the targets using BLAST approach and elimination of targets displaying homologies to other human genes, four target sequences were selected with starting bases located at positions 572, 1124, 1626, and 2858, respectively. The corresponding 19-nt double-stranded siRNAs with 3′-dithymidine overhangs were synthesized (Qiagen). The sequences and target locations of four PAR-1 siRNAs are listed in Table 1.

Cell transfection. A double transfection protocol [6] was employed to efficiently deliver siRNAs to DU 145 cells. Briefly, different concentrations of siRNA were mixed with RNAiFect reagent (Qiagen) to obtain the transfection complexes, which were added to 8×10^4 cells in complete medium. Cell suspensions were seeded into 12-well plates and left for 5 h, followed by replacement of the transfection medium with fresh complete medium. On the following day, adherent cells were re-transfected with siRNA using the same conditions as for transfection performed in cell suspensions. A scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) not inducing specific degradation of known cellular mRNA was used as control.

Semiquantitative RT-PCR. Cellular RNA was isolated at different time points after thrombin treatment or siRNA transfection using TRIzol reagent and digested with RQ1 RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA was reverse-transcribed using the Superscript II preamplification system (Life Technologies). Duplex PCRs were performed as previously reported [6]. 0.04 μM of each glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (predicted product size 305 bp) [6] and 0.2 μM of each primers for IL-8 (466 bp) [23], VEGF (249 bp) [24], or PAR-1 (598 bp) [6] were added to the same PCR tube. 25–30 cycles were performed and PCR products were electrophoresed on 2% agarose gels. The intensities of PCR products were analyzed using the ImageJ 1.28 u package (NIH, Bethesda, MD, USA).

Flow cytometric analysis of PAR-1 protein. After 72 h of the initial siRNA transfection, DU 145 cells were analyzed for cell surface PAR-1 expression using a mouse monoclonal anti-human PAR-1 IgG1 (SPAN12, Beckman Coulter Immunotech, Krefeld, Germany), which recognizes intact PAR-1 molecules by flow cytometry as previously described [6].

Immunoassay for IL-8 and VEGF. The concentrations of IL-8 and VEGF in cell culture supernatants were determined using sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Results were presented as means ± SEM. Data were analyzed using Student's two-tailed t test to assess differences which were considered significant at $P < 0.05$.

Results

Stimulation of IL-8 and VEGF by thrombin

Thrombin-induced IL-8 and VEGF expression in growth-arrested DU 145 cells (Fig. 1). IL-8 release was stimulated by thrombin treatment for 3–48 h as compared to untreated cells with a maximal response at 6 h treatment time in the presence of 1.0 U/ml thrombin (Fig. 1A). Thrombin induced less pronounced changes in VEGF expression during the initial 3–6 h treatment (Fig. 1B).

Table 1
Design of small interfering RNAs (siRNAs) targeting human protease-activated receptor 1 (PAR-1, gi 6031164)

siRNA No.	Sequence	DNA target sequence	Target nucleotide position
1	Sense 5′-GGG ACU GCU GGG AGG UUA AdTdT-3′ Antisense 3′-dTdTCCC UGA CGA CCC UCC AAU U-5′	5′-AA GGG ACT GCT GGG AGG TTA A-3′	1626–1646
2	Sense 5′-GCA GUC CUC UUC AAA AAC AdTdT-3′ antisense 3′-dTdTCGU CAG GAG AAG UUU UUG U-5′	5′-AA GCA GTC CTC TTC AAA AAC A-3′	572–592
3	sense 5′-CCC UGC UCG AAG GCU ACU AdTdT-3′ antisense 3′-dTdTGGG ACG AGC UUC CGA UGA U-5′	5′-AA CCC TGC TCG AAG GCT ACT A-3′	1124–1144
4	sense 5′-ACA AGG CCU GUC AGC UAA AdTdT-3′ antisense 3′-dTdTUGU UCC GGA CAG UCG AUU U-5′	5′-AA ACA AGG CCT GTC AGC TAA A-3′	2858–2878

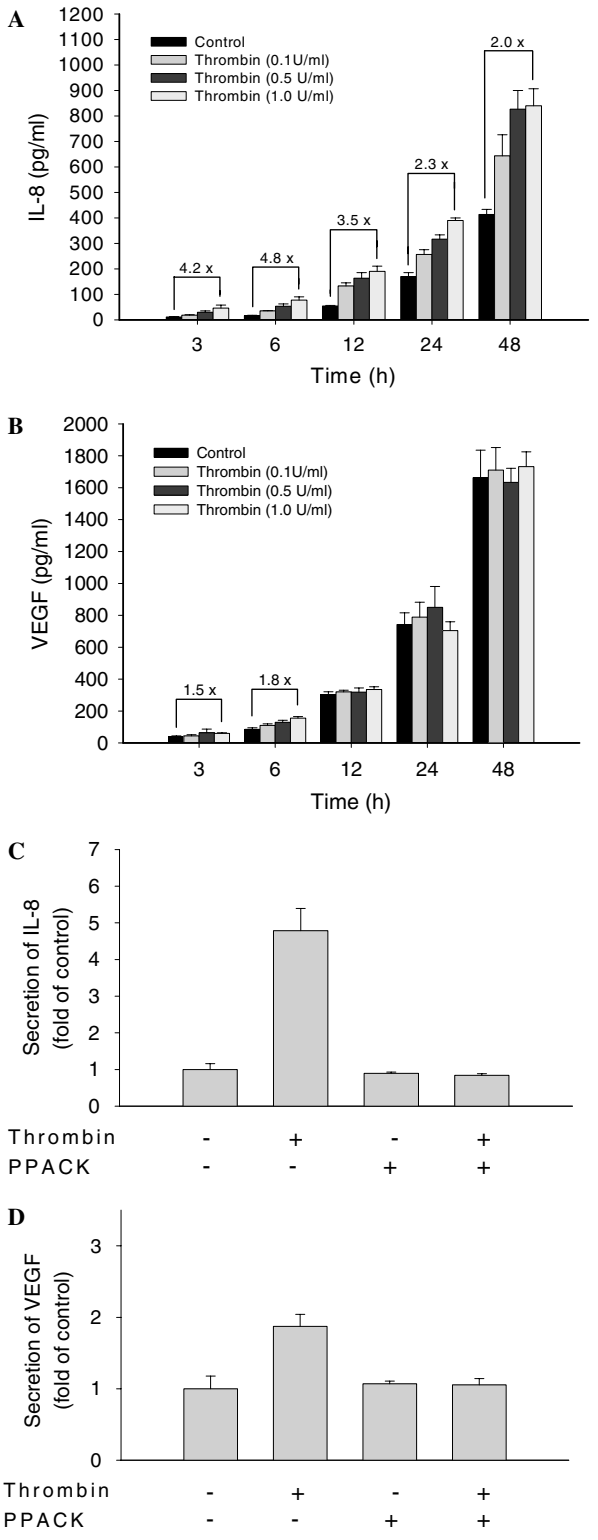

Fig. 1. Time- and dose-dependent thrombin stimulation of IL-8 and VEGF proteins from DU 145 cells. Growth-arrested DU 145 cells were treated with different concentrations of thrombin in serum-free medium. Cell culture supernatants were collected at the time points indicated, and concentrations of IL-8 (A) and VEGF (B) were determinated by ELISA. PPACK pretreatment of thrombin (1.0 U/ml) abolished the upregulation of IL-8 (C) and VEGF (D) secretion after 6 h of thrombin treatment. Data are from three to four experiments with duplicate samples throughout.

Afterward, no significant effect of thrombin on VEGF expression was observed. Similar to thrombin-induced IL-8 secretion, a maximal increase of VEGF secretion was noted after 6 h stimulation with 1.0 U/ml thrombin. Therefore, this treatment was selected for additional experiments except where stated otherwise.

The formation of PPACK-thrombin complex inhibits thrombin's proteolytic activity without interfering with thrombin binding to cell surfaces [25]. Accordingly, PPACK-pretreated thrombin was devoid of amidolytic activity as assessed by a chromogenic substrate S-2238 (data not shown). PPACK pretreatment completely abolished thrombin-induced IL-8 and VEGF expression (Figs. 1C and D), suggesting that thrombin's proteolytic activity is essential for the observed effects.

Changes in IL-8 and VEGF mRNA levels were less pronounced compared to the changes in protein concentrations (Fig. 2). The IL-8 transcript level was increased by 40–70% after 1.0 U/ml thrombin treatment for 1–4 h (Figs. 2A and C). In addition, thrombin elicited only 20% induction of VEGF mRNA levels after 0.5–4 h treatment (Figs. 2B and D).

Target sequence- and concentration-dependent PAR-1 gene silencing by RNA interference

Double transfection of DU 145 cells with siRNAs resulted in highly suppressed PAR-1 transcription levels 48 h post the initial transfection when used 50 nM of any of the four siRNAs tested (Fig. 3A). PAR-1 siRNA No. 3 revealed maximal inhibitory effect leading to undetectable PAR-1 mRNA. The siRNA-mediated gene silencing was concentration-dependent (Fig. 3B). The scrambled siRNA did not interfere with PAR-1 mRNA expression.

Flow cytometric analysis performed 72 h after transfection demonstrated that the expression of PAR-1 protein at the cell surface was significantly inhibited in cells transfected with PAR-1 siRNA No. 3 compared to cells transfected with scrambled siRNA (Fig. 3C).

Reversal of thrombin-induced cytokine expression by PAR-1 silencing

siRNA-transfected DU 145 cells, after overnight growth arrest, were incubated with 1.0 U/ml thrombin. In contrast to the cells treated with scrambled siRNA which remained responsive to thrombin with induction of IL-8 and VEGF secretion, PAR-1 siRNA No. 3 transfection significantly reversed the thrombin-induced cytokine secretion (Fig. 4), providing evidence that thrombin's effects are mediated by its cellular receptor.

Effects of ERK and PI3K inhibitors on thrombin-induced IL-8/VEGF expression

Preincubation of growth-arrested DU 145 cells with the ERK inhibitor PD98059 followed by the addition of

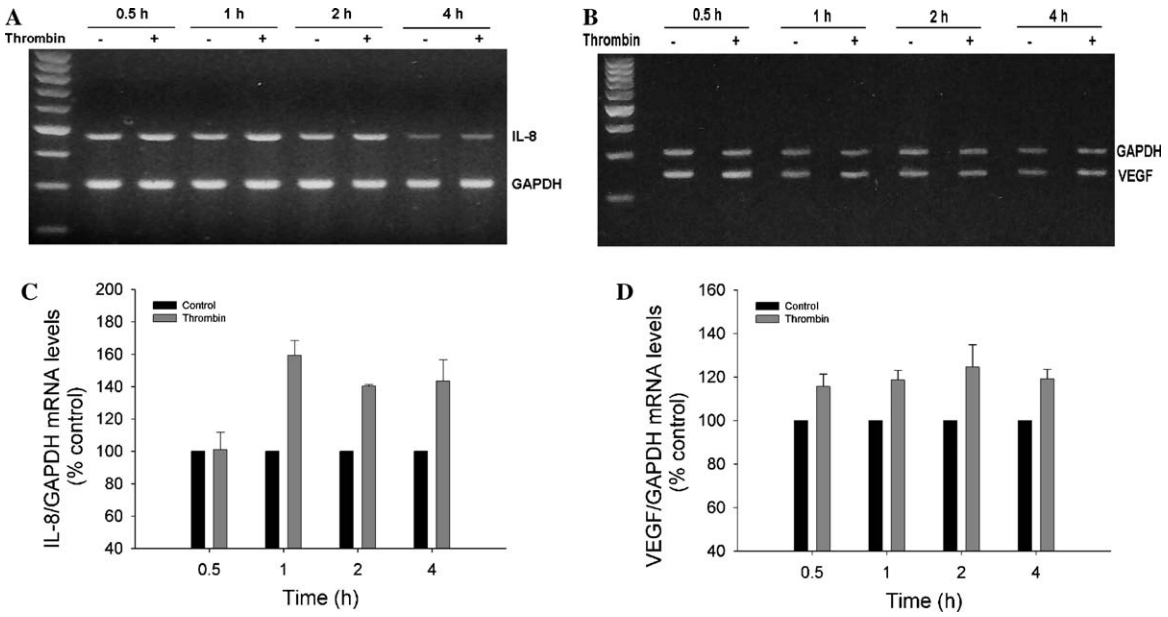

Fig. 2. Induction of IL-8 and VEGF mRNA levels in DU 145 cells by thrombin. Representative duplex RT-PCR analysis of IL-8 (A) and VEGF mRNA (B) from two individual experiments after thrombin (1.0 U/ml) stimulation for 0.5–4 h. GAPDH amplification was used as loading control. Quantitative data of mRNA levels of IL-8 (C) and VEGF (D) from RT-PCR assays.

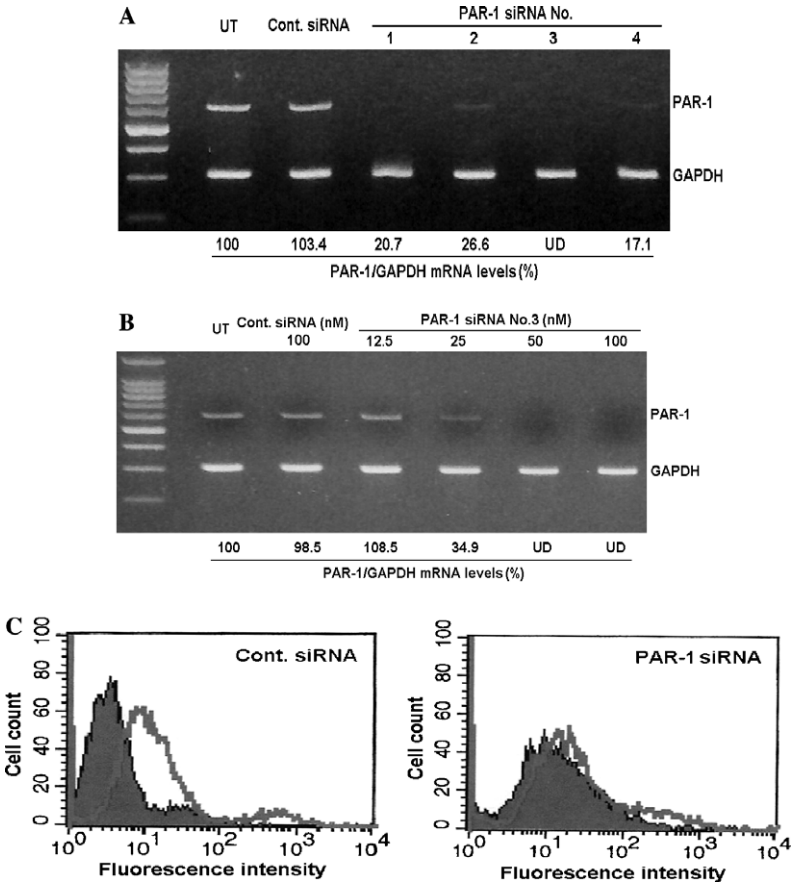

Fig. 3. siRNA-mediated suppression of PAR-1 expression in DU 145 cells using a double transfection protocol. (A) Each of four synthetic siRNAs against different targets was delivered into cells at 50 nM concentration. (B) PAR-1 siRNA No. 3 was applied at different concentrations. A scrambled siRNA was used as control for PAR-1 gene silencing experiments (UT, untreated; UD, undetectable). Residual PAR-1 mRNA levels were assessed by RT-PCR analysis 48 h after the initial transfection. (C) Seventy-two hours post the initial transfection, flow cytometry was used to examine PAR-1 protein expression on cell surface. Cells were harvested and stained for PAR-1 molecules using the monoclonal antibody SPAN12 (bold line) and isotypic mouse IgG was performed as negative control (filled graph).

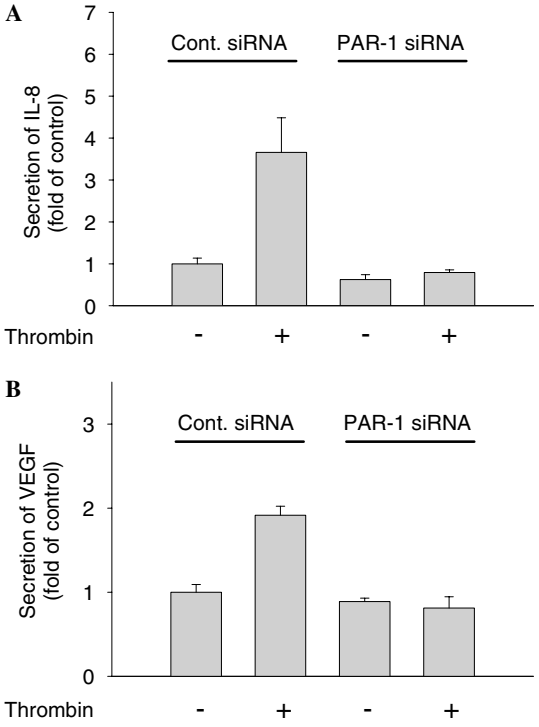

Fig. 4. Suppression of PAR-1 expression by siRNA reversed thrombin-induced IL-8 and VEGF expression. Double-transfected cells with either PAR-1 siRNA No. 3 or a scrambled siRNA were treated with 1.0 U/ml thrombin for 6 h. Secretion of IL-8 (A) and VEGF (B) was determined by ELISA. Three individual experiments were performed with duplicate samples per experiment.

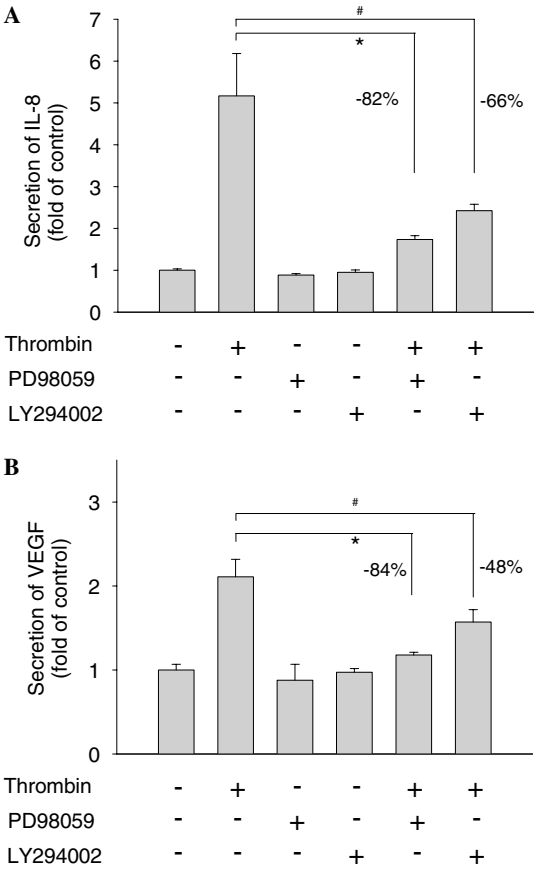

Fig. 5. Involvement of ERK (panel A) and PI3K (panel B) pathways in thrombin-induced expression of IL-8 and VEGF from DU 145 cells. Either the specific ERK inhibitor PD98059 (20 μM) or the specific PI3K inhibitor LY294002 (25 μM) was applied to growth-arrested cells before thrombin addition with continuous presence of the inhibitor. After 6 h of thrombin treatment, cell culture supernatants were harvested and the cytokine concentrations were assayed by ELISA. Data are derived from three separate experiments with duplicate samples per experiment. ($^{*}P < 0.05$; $^{\#}0.05 < P \leqslant 0.10$ versus thrombin-treated cells in the absence of inhibitor).

thrombin in the presence of the inhibitor for 6 h caused an 82.3% reduction of IL-8 secretion elicited by thrombin ($P = 0.028$). PD98059 treatment also inhibited the stimulatory effect of thrombin on VEGF secretion (83.9% decrease, $P = 0.011$). Pretreatment with the PI3K inhibitor LY294002 yielded similar effects with inhibition of thrombin-induced secretion of IL-8 and VEGF by 65.8% ($P = 0.056$) and 48.5% ($P = 0.10$), respectively (Fig. 5).

Discussion

Overexpression of several proangiogenic factors including VEGF, IL-8, basic fibroblast growth factor (bFGF), and angiopoietins has been demonstrated to be critically involved in the development and progression of cancer in general [26] as well as specifically for prostate cancer [27]. We observed that DU 145 prostate cancer cells secreted increased amounts of VEGF and IL-8 in comparison to PNT1A immortalized prostate epithelial cells (data not shown), supporting the conceptual view that VEGF and IL-8 levels are linked to prostate malignancy.

IL-8 and VEGF act as autocrine/paracrine cytokines in the tumor and its surrounding microenvironment. A recent study reported increased expression of IL-8 and its cellular receptors (CXCR1 and CXCR2) in prostate cancer tissues. Moreover, the activation of IL-8 receptors induced PC-3 prostate cancer cell proliferation in vitro [28]. Similarly, increased expression of VEGF and its receptors (VEGFR-1 and VEGFR-2) has been observed in prostatic intraepithelial neoplasia and malignant cells [29]. However, another study reported reduced expression of VEGFR-1 on prostate cancer cell membranes [30]. At present, the association between VEGF receptors and prostate cancer progression remains unknown. Soluble VEGFR-1 (sVEGFR-1) formed by alternative splicing of VEGFR-1 is known to prevent VEGF signaling on transmembrane VEGFR-1 [31]. We observed that DU 145 cells did not secrete sVEGFR-1 in culture, suggesting that the inhibitory control of VEGF by sVEGFR-1 is not in place in this cellular model. Furthermore, thrombin treatment did not elicit secretion of sVEGFR-1 (data not shown).

The combined treatment with coagulation factor VIIa and tissue factor specifically enhanced IL-8 expression via trypsin receptor PAR-2 in MDA-MB-231 breast carcinoma cells. However, thrombin failed to induce IL-8 in this cell type which also expressed PAR-1 [18]. Thrombin-catalyzed fibrin formation was detectable in oral squamous cell

carcinoma in vivo. Incubation with fibrin caused a stimulation of IL-8 expression, while thrombin itself did not elaborate the upregulation of IL-8 in these cells [22].

The present study provided evidence that thrombin directly stimulated IL-8 expression and secretion from DU 145 prostate cancer cells in vitro. Thrombin even at low concentration (0.1 U/ml) induced a rapid IL-8 release (3–6 h), suggesting that small amounts of thrombin are sufficient to rapidly trigger the release of the proangiogenic cytokine. We also showed that thrombin at 0.5–1.0 U/ml induced VEGF production in a short-term period (up to 6 h). Longer periods of thrombin treatment did not lead to further induction of VEGF secretion in comparison to untreated cells. Our results demonstrated that thrombin had more potent effect on IL-8 synthesis compared to VEGF in DU 145 cells. Thrombin's proteolytic activity was essential for its stimulatory effects on cytokine release since PPACK completely neutralized the effects observed.

We have previously established a novel siRNA transfection protocol with the initial transfection in cell suspensions and a second transfection performed on cell monolayers, which caused highly efficient gene silencing using a commercially available PAR-1 siRNA [6]. In the present study, four siRNA species designed against different target sites of PAR-1 gene were applied to suppress PAR-1 expression and the most potential siRNA target sequence was identified. It has been observed that DU 145 cells express PAR-1 and the trypsin receptor PAR-2 while the additional thrombin receptors PAR-3 and -4 were undetectable [5,32]. The present study demonstrated that suppression of PAR-1 expression as verified at both the mRNA and protein levels reversed the induction of IL-8 and VEGF expression elicited by thrombin, demonstrating the essential and exclusive role of PAR-1 for thrombin's effects in DU 145 cells.

Thrombin-induced PAR-1 activation elicits complex signaling events resulting in modifications of cell shape, proliferation, migration, adhesion, and protein expression [8]. An array of diverse kinase signaling patterns involved in IL-8 or VEGF regulation have been demonstrated. For example, IL-8 upregulation by activation of N-CAM-related kinase in DU 145 cells was mediated by MEK/ERK but not PI3K/AKT pathway [33]. Arsenite enhanced VEGF secretion from DU 145 cells via the PI3K/AKT pathway [34]. Thrombin induced the expression of VEGF in FS4 fibroblasts with a PI3K- and serine/threonine kinase-dependent but ERK-independent pattern [7]. Results from the present study suggested that both ERK and PI3K pathways, may be involved in thrombin-induced angiogenic cytokine expression in DU 145 prostate cancer cells because either of the inhibitors partly inhibited the effects. The ERK inhibitor demonstrated more pronounced inhibitory effects compared to the PI3K inhibitor, lending support to the notion that the ERK pathway may play a more critical role in mediating thrombin-induced angiogenic cytokine expression in the cells under consideration.

In conclusion, our results demonstrated that the thrombin/PAR-1 system stimulated the expression and secretion of IL-8 and VEGF from DU 145 human prostate cancer cells in vitro with more pronounced effects on IL-8. Both, the ERK and PI3K pathways may be involved in thrombin-induced expression of these two proangiogenic cytokines. The data suggest a critical thrombin/PAR-1 interface as related to the growth and angiogenesis of prostate cancer. Due to the highly complex intracellular molecular mechanisms of thrombin signaling, the suppression of its transmembrane receptor(s) using a RNAi approach may form part of an overall strategy to control tumor progression in prostate cancer.

Acknowledgments

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