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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · FEBRUARY 2010

Impact Factor: 5.09 · DOI: 10.1002/ijc.24904 · Source: PubMed

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Kallikrein-related peptidase-4 initiates tumor–stroma interactions in prostate cancer through protease-activated receptor-1

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In prostate cancer, the mechanism by which the stromal cells surrounding the cancer epithelium become reactive and overproduce growth factors is unclear. Furthermore, the precise process of how these stromal cells stimulate the cancer epithelium is not fully understood. We recently found that protease-activated receptor-1 (PAR-1) in these reactive stromal cells is upregulated. To investigate the role of PAR-1 in the stromal–epithelial interaction, WPMY-1 stromal myofibroblasts were stimulated with PAR-1 agonists including thrombin and PAR-1 activating peptide. We show that WPMY-1 cells have functional PAR-1 by signaling through ERK1/2. Conditioned media (CM) from PAR-1 agonists-treated WPMY-1 cells stimulate the epithelial LNCaP cells leading to ERK1/2 activation and cell proliferation. Cytokine array analysis of the CM demonstrates that PAR-1 induces stromal cells to release numerous cytokines, of which interleukin 6 (IL-6) is the major factor responsible for mitogenic signaling in LNCaP cells. CM further induces expression of prostate-specific kallikrein-related peptidase-3 (KLK3/PSA) and KLK4 in LNCaP cells *via* the IL-6 pathway. Moreover, KLK4 functions as a potent agonist of PAR-1 by cleaving the receptor at the proper site on cell surface. KLK4 triggers transmembrane signaling and upregulates IL-6 in WPMY-1 cells through PAR-1. Immunohistochemical analysis indicates that PAR-1 is predominantly expressed in peritumoral stroma while KLK4 is produced exclusively by the epithelial cancer cells. These data provide evidence for a novel double-paracrine mechanism whereby cancer epithelium produces KLK4 to activate PAR-1 in the surrounding stroma, which in-turn releases cytokines (IL-6) that stimulate cancer cells to proliferate and increase production of KLKs.

Fast Track

Although prostate cancer is the most commonly diagnosed malignancy of men,[1] the underlying causes of its lethal progression remain incompletely understood. The progression of prostate cancer from an androgen-dependent (AD) to androgen-independent (AI) state involves multistep processes mediated in part by tumor–stroma interactions. More specifically, the stromal cells immediately adjacent to cancer cells may become reactive and release high levels of growth factors contributing to the AI state. For example, stromal insulin-like growth factor-1 (IGF-1) can activate the androgen receptor (AR) in the absence of androgen.[2] Other potential AR activators include epidermal growth factor (EGF), forskolin (FSK), keratinocyte growth factor (KGF) and interleukin 6 (IL-6).[2–4] The precise mechanisms that regulate these stromal growth factors are not clearly known, but may involve stimulation from epithelial cancer cells. Presumably, prostate cancer cells may activate stromal cells, which in turn may stimulate the cancer cells to become more aggressive.

The molecular mechanisms of tumor–stroma interaction are very complex and could be mediated by cell–cell contact, cell–matrix interplay or cell-soluble factor communication.[5] For example, it is clear that prostate tumor growth *in vivo* is markedly accelerated by the presence of organ-specific stromal cells.[6,7] Recent evidence also suggests that the interactions mediated by fibroblast-derived growth factors appear to be particularly critical in facilitating tumorigenesis.[8] Stromal cell-derived factor-1 (SDF-1), a factor secreted by fibroblasts, promotes breast cancer angiogenesis by recruiting endothelial progenitor cells.[9] SDF-1 also increases tumor growth directly by activating its receptor CXCR4 on cancer cells.[9] In prostate cancer, stromal transforming growth factor-β1 (TGF-β1) upregulates epithelial CXCR4 expression, allowing fibroblast-derived SDF-1 to activate the phosphoinositide-3-kinase/Akt signaling pathway in cancer cells.[10] Therefore, fibroblasts seem to be the prominent modifier of cancer progression through secretion of numerous growth factors which work in tandem to promote tumor growth.[8]

Before the stromal fibroblasts can increase expression of growth factors, they may require stimuli such as those from epithelial cancer cells. Recent evidence suggests that proteases from cancer cells may function as modulators of tumor–

Key words: KLK4, PAR-1, IL-6, prostate cancer, signaling
Grant sponsor: NIH-Prostate Cancer Program Project; **Grant number:** 5P01CA085859-05; **Grant sponsor:** SMT Foundation
DOI: 10.1002/ijc.24904
History: Received 2 Apr 2009; Accepted 21 Aug 2009; Online 30 Sep 2009
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stroma interactions.[11] In prostate, the epithelium produces several proteases that are members of the tissue kallikrein (kallikrein-related pepdidases, KLKs) family[12,13]: KLK2, KLK3 (PSA) and KLK4. The suggested function of these 3 KLKs in cancer progression includes degradation of ECM, and direct or indirect activation of mitogenic proteins such as latent TGF-β.[14,15] More recently, kallikreins have been shown as potential activators for protease-activated receptors (PARs),[16–18] which are G-protein-coupled-receptors implicated in various cancers.

PARs (PAR-1, -2, -3 and -4) are tethered-ligand receptors that are activated *via* cleavage of their extracellular amino terminus by serine proteases.[19,20] Once activated, PARs trigger a cascade of downstream biological events, leading to diverse cellular responses such as cell proliferation, tissue repair and gene transcription.[19,20] For example, PAR-1 and PAR-2 promote colon cancer[21,22] and gastric cancer[23] cell proliferation by transactivating epidermal growth factor receptor (EGFR). It is noted that the signaling role of PAR-3 remains unclear. PAR-3 does not signal in response to its tethered ligand-derived peptide or to thrombin,[24] but it may regulate other PAR signaling by receptor dimerization.[25] In prostate cancer, PAR-1 and PAR-2 stimulation activates RhoA and Rac1/Cdc42 signaling with resultant cytoskeletal changes and enhanced migration in LNCaP cells.[26,27] PAR-1 and PAR-2 also can upregulate oncogenic genes such as vascular endothelial growth factor (VEGF).[28] *In vivo*, inducible PAR-1 expression in rat prostatic carcinoma significantly enhances both angiogenesis and tumor growth.[29] Although PAR-1, PAR-3 and PAR-4 are activated mainly by thrombin and PAR-2 by trypsin, emerging evidence suggests that organ-specific serine proteases may be physiologically important.[20] Recently, we as well as others have found that KLK4 is a potential activator of PAR-1 and PAR-2.[16,18] We also observed that PAR-1 is characteristically localized in the peritumoral stromal cells.[26,30] This suggests that stromal PAR-1 might be an important receptor involved in cancer kallikrein-mediated activation of stromal cells.

In the current study, we explored the possibility that epithelial cancer cells can stimulate stromal cells through PAR-1 activation resulting in release of growth factors and cytokines, which sequentially drive cancer growth. In particular, KLK4, which is exclusively produced by prostate epithelium, activates PAR-1 on prostate stromal cells. The resultant conditioned media of the stromal cells stimulate prostate cancer cells, mainly through IL-6. Therefore, both KLK4 and PAR-1 appear to be involved in a "double-paracrine" process between prostate cancer cells and the surrounding stromal cells.

Material and Methods

Reagents

Antibodies were obtained as follows: vimentin (Vim3B4) from DakoCytomation (Glostrup, Denmark); α-smooth muscle actin (1A4) from Sigma (St. Louis, MO); CD90 fibroblast antigen (AS02) from EMD Biosciences (San Diego, CA); PAR-1 (ATAP2), GAPDH (V-18), PSA (C-19), KLK4 (N-14), IL-6 (H-183) from Santa Cruz Biotechnology (Santa Cruz, CA); p44/42 MAPK and phospho-p44/42 MAPK (E10) from Cell Signaling Technology (Danvers, MA). Human α-thrombin was from Enzyme Research Labs (South Bend, IN). FITC-labeled streptavidin and bicinchoninic acid (BCA) protein assay kit were from Pierce Chemical Company (Rockford, IL). Amidated PAR-1 activating peptide (TFLLRN) was from Anaspec (San Jose, CA). U0126 was from Cell Signaling. Human IL-6 ELISA kit was from BD Biosciences (San Jose, CA). Doxycycline, D-biotin, bovine serum albumin and paraformaldhyde were from Sigma. Tetracycline-free fetal bovine serum was from Clontech (Mountain View, CA). Human recombinant IL-6 and IL-8 were from R&D Systems (Minneapolis, MN), and GROα, GROβ, GROγ and MCP-1 were from PeproTech (Rocky Hill, NJ).

Cell culture

LNCaP cells from American Type Culture Collection (ATCC) were cultured as previously described.[27] WPMY-1 cells from ATCC were cultured in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. Chinese hamster ovary Tet-On (CHO-TO) cells stably expressing an enzymatically biotinylated PAR-1 were established and cultured as described.[31]

Recombinant KLK4

Human recombinant KLK4 was purified and characterized as previously described.[15]

Flow cytometry

WPMY-1 cells grown to 80% confluence were analyzed as previously described.[27] Primary monoclonal antibodies against PAR-1, vimentin, α-smooth muscle actin and CD90 fibroblast antigen were used at 1:100 dilution and the FITC-conjugated anti-mouse IgG was at 1:400 dilution. IgG isotype control antibody or secondary antibody alone was used as control. The washed cells were analyzed on a Becton Dickinson FACScan (San Jose, CA) flow cytometer.

ERK1/2 phosphorylation

At 80% confluence, the cells were washed with PBS and switched to serum-free medium overnight. The cells were washed again and cultured with fresh serum-free medium for 2 hr and then treated with agonists for the indicated times. After treatment, the cells were washed with cold TBS and lysed with RIPA [50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with 30 nM DNase and protease inhibitors cocktail. Cell lysates were gently shaken for 10 min at 4°C and then clarified by centrifugation, and protein concentration was determined using the BCA protein assay. Samples containing equal amounts of protein were analyzed by

Western blot to detect p44/42 MAPK (T-ERK1/2) or phosphorylated p44/42 MAPK (P-ERK1/2) as described.[18,32]

Preparation of conditioned medium

WPMY-1 cells were seeded in 15-cm culture dishes at 1.6×10^6 cells/dish with complete medium. After reaching 70% confluence, cells were washed with PBS and switched to serum-free medium overnight. Cells were then washed again and treated with or without agonists in serum-free medium for 24 hr. Conditioned medium was collected, centrifuged and stored at −70°C. For IL-6 antibody neutralization, 1 μg ml^{-1} of anti-IL-6 antibody was added to the conditioned medium and shaken for 15 min at 4°C and then incubated at 37°C water bath for 30 min with occasional mixing.

Cell proliferation

LNCaP cells were seeded in 96-well culture plates at 1×10^4 cells/well in 100 μl RPMI containing 0.5% charcoal-stripped FBS.[33] Twenty-four hours later, conditioned medium from WPMY-1 cells was added directly to LNCaP cells without removal of the old medium. Cell proliferation was assayed 72 hr later using a Quick Cell Proliferation Assay Kit (BioVision, Mountain View, CA) as described.[32] Experiments were done with 6 wells per treatment and repeated once.

Cytokine array analysis

Cytokine analysis was performed using RayBio Human Cytokine Antibody Array 5 (RayBiotech, Norcross, GA). The 79 cytokine antibodies arrayed on membranes were blocked for 30 min, and incubated with 1 ml of conditioned medium for 2 hr at room temperature. After washing, membranes were incubated with biotin-conjugated antibodies at 4°C overnight, and then with HRP-streptavidin for another 2 hr at room temperature. Detection was done according to the manufacturer's instruction.

Immunoassay of IL-6

WPMY-1 cells were seeded into 6-well plates (1.2×10^5 cells/well) with complete medium. After reaching 80% confluence, cells were washed with PBS and incubated in serum-free medium for 24 hr. The medium was then replaced with fresh serum-free medium containing various agonist and/or inhibitors for 24 hr. Levels of IL-6 in the CM were determined with an IL-6 ELISA kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions.

PAR-1 cleavage by thrombin or KLK4 on intact cells

Stable CHO-TO cells expressing PAR-1 were established as described.[31] Twenty four hours prior to harvest, biotinylated PAR-1 expression was induced by addition of 50 μM D-biotin and 2 μg ml^{-1} doxycycline. Cells expressing biotinylated PAR-1 were harvested by washing twice with PBS and detaching the cells from the plate using 0.5 mM EDTA in PBS. Cells were collected in serum-free medium and sedimented at 500*g* for 3 min. Cells were then resuspended in serum-free medium, aliquoted and treated for 10 min at 37°C with either 10 nM thrombin, 100 nM recombinant KLK4, or with serum-free medium only. Cells were washed twice with 1 mM EDTA/PBS, then either stained with streptavidin-FITC (1:500) for detection of the biotin tag, or stained with PAR-1 antibody (ATAP-2) for detection of a PAR-1 epitope SFLLRNPNDKYEPF, which is located right after thrombin cleavage site LDPR/S.[34] The cells were fixed in 0.5% paraformaldehyde in PBS and analyzed using a flow cytometer as described above.

Transfection of small interfering RNA

Transfection of WPMY-1 cells with small interfering RNA (siRNA) was performed as previously described.[18] Briefly, WPMY-1 cells were transfected at 35,000 cells/well in 24-well plate with 75 nM SMARTpool® PAR-1 siRNA (Dharmacon, Lafayette, CO), p44/42 MAPK (ERK1/2) siRNA (Cell Signaling Technology) or GAPDH siRNA with 0.5 μl DharmaFECT® 1. Cells were treated with a second transfection 24 hr later, and then incubated for another 24 hr. ERK1/2 expression was analyzed by Western blot, and PAR-1 expression was analyzed using flow cytometry. For quantification of IL-6 levels, the cells were shifted to serum-free medium 48 hr after the second siRNA transfection and then treated with agonists as described above.

Immunohistochemistry

Primary prostate cancer tissues ($n = 12$) were obtained from the University of Washington Medical Center with approval from the Human Subject Division of the University of Washington. Serial sections (3 μm) were deparaffinized and rehydrated in xylene and a series of graded alcohols, and immunohistochemistry (IHC) with primary antibodies against PAR-1 and KLK4 was performed as previously described.[32] IgG at the same concentration as the corresponding primary antibodies was used as negative control.

Results

WPMY-1 cells express functional PAR-1

WPMY-1 is a human prostatic stromal cell line that was derived from a primary stromal culture and immortalized using the SV40 large T antigen.[35] Here, we performed flow cytometry to further characterize this cell line. As shown in Figure 1*a*, WPMY-1 cells coexpressed vimentin (a marker for cells of mesenchymal origin),[36] α-smooth muscle actin and CD90 (a specific fibroblast antigen).[37] These data prove that WPMY-1 is a stromal myofibroblast cell line. Furthermore, WPMY-1 cells expressed PAR-1.

To examine PAR-1 signaling in WPMY-1 cells, the cells were stimulated with PAR-1 agonists, and phosphorylation of ERK1/2 was measured as a readout by Western blot. Thrombin or PAR-1 activating peptide (PAR-1 AP) generated robust phosphorylated-ERK1/2. Maximal signal was achieved 5 min after PAR-1 stimulation and gradually decreased over 60 min (Fig. 1*b*). These data demonstrate that functional PAR-1

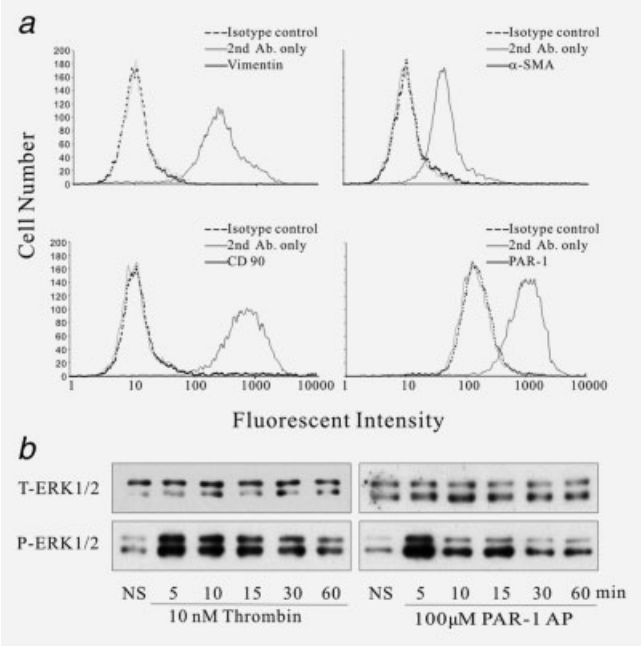


Figure 1. WPMY-1 cells express functional PAR-1. (*a*) Expression of vimentin, α-SMA, CD90 and PAR-1 was analyzed by flow cytometry (heavy line, primary antibody specific fluorescence; lightline, secondary antibody only; dotted line, isotype control). (*b*) WPMY-1 cells were stimulated with thrombin (10 nM) or PAR-1 AP (100 μM) for the time period indicated and ERK1/2 activation was measured by Western blot. NS: nonstimulated; T-ERK1/2: total-ERK1/2; P-ERK1/2: phosphorylated-ERK1/2.

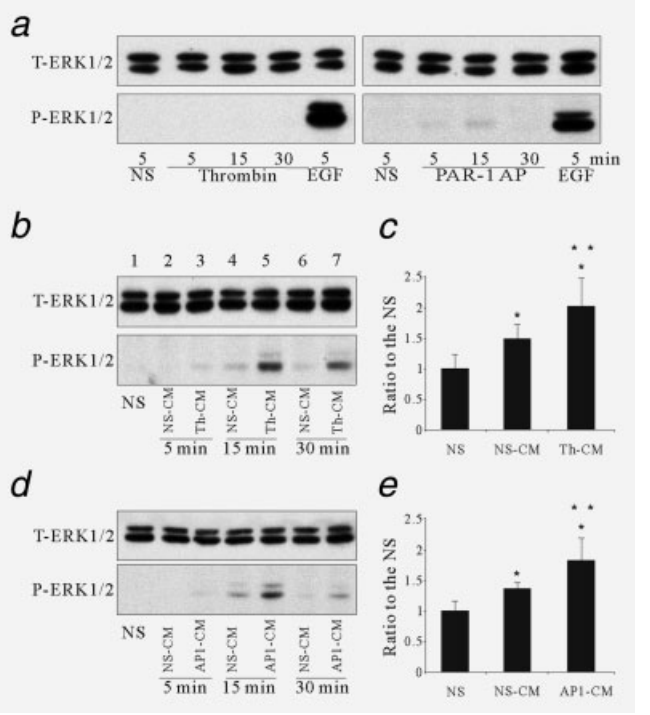


Figure 2. Conditioned medium from PAR-1 activated WPMY-1 cells stimulates ERK1/2 signaling in LNCaP cells. (*a*) LNCaP cells were stimulated with thrombin (10 nM), PAR-1AP (100 μM) or EGF (10 ng ml^{-1}) for the time period indicated and ERK1/2 activation was measured by Western blot. (*b*) WPMY-1 cells were untreated or treated with thrombin (10 nM) for 24 hr followed by collection of the conditioned medium (CM). LNCaP cells were stimulated with the CM for indicated time and ERK1/2 activation was measured by Western blot. (*c*) LNCaP cells were stimulated with or without the CM for 72 hr and cell proliferation was determined by WST assay. (*d*) WPMY-1 cells were untreated or treated with PAR-1 AP (100 μM) for 24 hr followed by collection of the conditioned medium (CM). LNCaP cells were stimulated with the CM for indicated time and ERK1/2 activation was measured by Western blot. (*e*) LNCaP cells were stimulated with or without the CM for 72 hr and cell proliferation was determined by WST assay. *Columns*, mean of sextuplicate; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the NS. **$p < 0.05$, compared to the NS-CM. NS-CM: CM derived from non-stimulated WPMY-1 cells; Th-CM: CM derived from thrombin-stimulated WPMY-1 cells; AP1-CM: CM derived from PAR-1 AP stimulated WPMY-1 cells.

is expressed in WPMY-1 stromal myofibroblast cells. PAR-1 activation did not signal through other MAPK pathways including p38MAPK and JNK in stromal cells (data not shown); we therefore focused on the mechanism of PAR-1-ERK1/2 signaling mediated tumor–stromal interactions.

Conditioned medium from thrombin-activated WPMY-1 cells (thrombin-CM) stimulates ERK1/2 signaling in LNCaP cells

We recently found that PAR-1 is upregulated in prostate reactive stroma surrounding cancer epithelial cells.[30] Accordingly, we investigated whether PAR-1 activation of stromal cells can relay a paracrine effect back to the cancer cells. WPMY-1 cells were treated with the PAR-1 agonist thrombin for 24 hr and the resultant conditioned medium (thrombin-CM) was used to activate the LNCaP cells. Direct stimulation of LNCaP cells with PAR-1 agonists did not generate any phosphorylated-ERK1/2, but intact signaling with epidermal growth factor (EGF) was present (Fig. 2*a*). However, treatment of LNCaP cells with the thrombin-CM (Th-CM) from WPMY-1 cells induced a time-dependent phosphorylated-ERK1/2 with a maximum effect at 15 min (lanes 3, 5, 7, Fig. 2*b*). Pretreatment of the thrombin-CM with the irreversible thrombin inhibitors hirudin or P-PACK did not reduce its mitogenic effect (data not shown). Furthermore, the thrombin-CM from WPMY-1 cells also stimulated LNCaP cell proliferation (Fig. 2*c*). Similar results were obtained when the PAR-1 AP resultant conditioned medium (AP1-CM) was used for LNCaP stimulation (Figs. 2*d* and 2*e*). These data indicate that PAR-1-mediated stromal stimulation of cancer epithelial cells depends on an unknown soluble growth factor(s) and/or cytokine(s) in the conditioned medium.

IL-6 is the major factor in the thrombin-CM responsible for LNCaP stimulation

To identify which factors are upregulated in the thrombin-CM isolated from WPMY-1 cells, we used a human antibody array that detects 79 cytokines (Fig. 3*a*). Compared to the nonstimulated CM, the factors that were significantly increased in the thrombin-CM included growth-related oncogene (GROs) [grid J1, Fig. 3*a*], IL-6 [H2], IL-8 [J2], and monocyte chemotactic protein-1 (MCP-1) [E3]. Other factors that were upregulated to a lesser extent included epithelial neutrophil activating peptide-78 (ENA-78) [G1], IL-3 [E2], IL-7 [I2], oncostatin M [A5], vascular endothelial growth factor (VEGF) [C5], brain-derived neurotrophic factor (BDNF) [F5], eotaxin [I5], fibroblast growth factor-7 (FGF-7) [C6], FGF-9 [D6] and osteoprotegerin [B8].

We then focused on these more potent cytokines to determine the major one(s) that stimulated the cancer cells. LNCaP cells were treated with recombinant GROα, GROβ, GROγ, IL-6, IL-8 and MCP-1, and assayed for ERK1/2 phosphorylation. As shown in Figure 3*b* (left), only recombinant IL-6 activated ERK1/2 in the LNCaP cells. Activation of ERK1/2 in LNCaP cells by recombinant IL-6 was time dependent with a maximal signal at 15 min (data not shown), which is comparable to the effect of thrombin-CM from WPMY-1 cells (Fig. 2*b*). Furthermore, neutralization of IL-6 by anti-IL-6 antibodies significantly blocked ERK1/2 signaling generated by the thrombin-CM (Fig. 3*b*, right). Taken together, these results support the hypothesis that IL-6 is the key cytokine from the thrombin-CM responsible for LNCaP stimulation.

The nature of IL-6 release in the thrombin-CM was then studied by ELISA. When treated with thrombin for 24 hr, WPMY-1 cells increased IL-6 production in a dose-dependent manner (Fig. 3*c*, left). IL-6 was also induced by PAR-1 AP, indicating that the thrombin-stimulated IL-6 production is mediated through PAR-1 (Fig. 3*c*, right). Furthermore, pretreatment with U0126, a pharmacological inhibitor of MEK, significantly inhibited IL-6 production induced by thrombin or PAR-1 AP (Fig. 3*c*, right). We further confirmed this result by RNA interference. The p44/42 MAPK (ERK1/2) siRNA effectively reduced ERK1/2 expression level in WPMY-1 cells (Fig. 3*d*, left). Under these conditions, ERK1/2 siRNA significantly knocked down IL-6 production induced by thrombin or by PAR-1 AP (Fig. 3*d*, right). Altogether, these data indicate that PAR-1-induced IL-6 production is mediated primarily by ERK1/2 activity.

Thrombin-CM induces PSA and KLK4 expression in LNCaP cells via an IL-6 mechanism

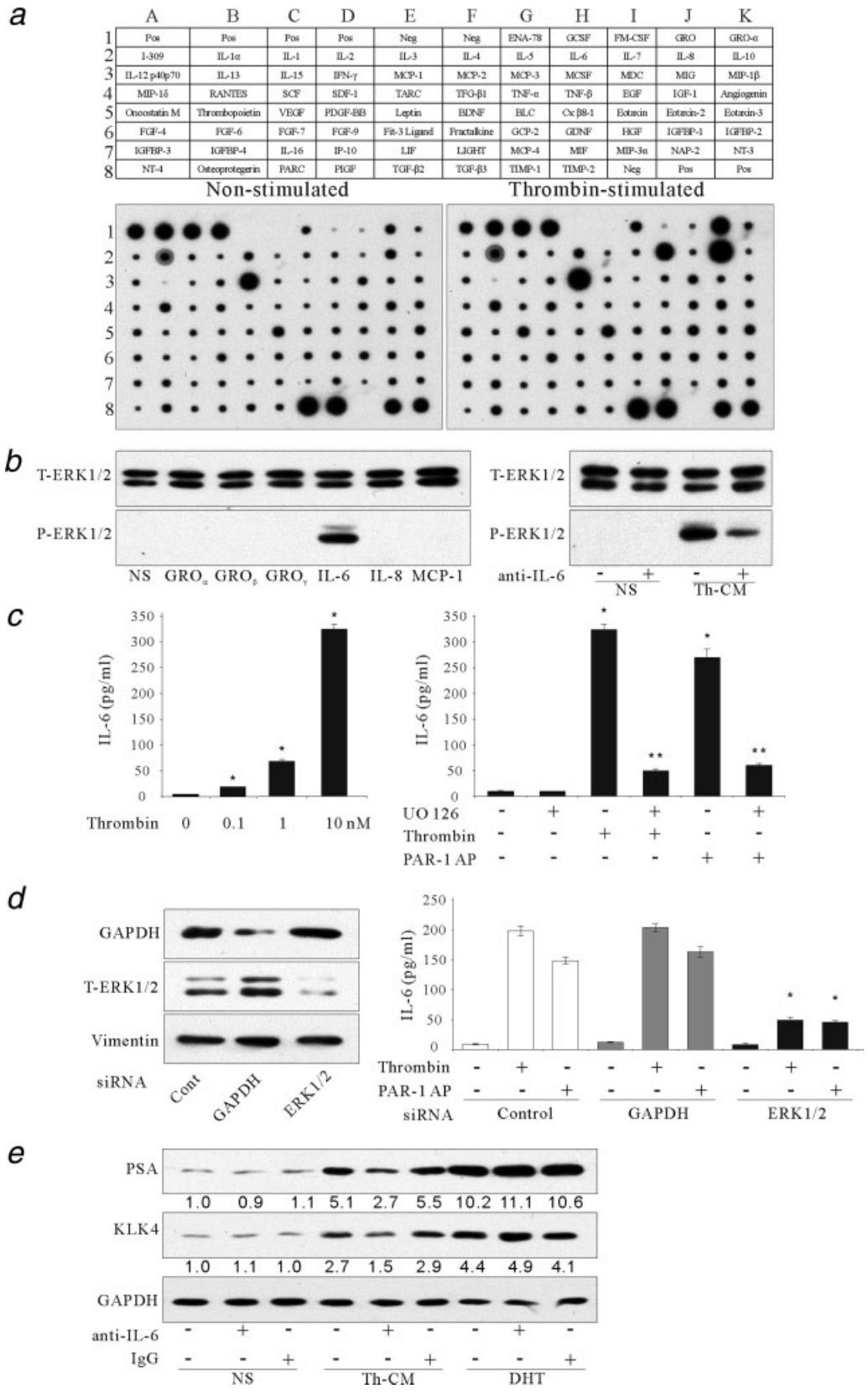
IL-6 is a pleiotropic cytokine implicated in tumor growth.[38] In prostate cancer, it has been well-documented that IL-6 can regulate prostate cancer cell growth, in part by androgen-independent activation of the AR.[39] Therefore, downstream effects of IL-6 may include the upregulation of PSA[40] and other PSA-like kallikreins through AR activation. As IL-6 is the major factor in the thrombin-CM responsible for LNCaP stimulation, we tested this possibility by measuring PSA and KLK4 in LNCaP cells. As expected, dihydrotestosterone (DHT) caused a robust increase of PSA and a moderate increase of KLK4 (Fig. 3*e*). In a similar manner, thrombin-CM from WPMY-1 cells also increased PSA and KLK4 production by LNCaP cells. Preincubation of the thrombin-CM with anti-IL-6 partially blocked the effect while preincubation with an IgG control antibody had no effect (Fig. 3*e*), demonstrating that IL-6 is the important mediator involved. These data indicate that stromal CM could induce the expression of PSA-like kallikreins in cancer cells *via* an IL-6 mechanism.

KLK4 cleaves PAR-1 at the activation site on intact cell surface

Because PARs are activated by a unique proteolytic mechanism, any protease capable of cleaving at the activating site can trigger receptor activation.[19] Therefore, PARs can transduce signals in response to multiple tumor-generated proteases.[41] In prostate cancer, abundant kallikreins are potentially endogenous PAR activators because of their trypsin-like activities. Here, we used CHO-TO cells which express PAR-1 containing a biotinylated N-terminus[31] to seek direct evidence that KLK4 can activate PAR-1 by specifically cleaving the activating site in the N-terminus (Fig. 4*a*). In these experiments, thrombin was used as a positive control for PAR-1 cleavage. Exposure of the cells to thrombin caused nearly complete loss of the N-terminal biotin-tag (Fig. 4*b*). Thrombin cleavage occurs at the N-terminal activation site of PAR-1 without nonspecific alternate cleavage(s). This was demonstrated by the lack of change in the levels of PAR-1 antibody binding domain (Fig. 4*c*). The biotin-tag was also cleaved by KLK4 while only causing a minimal loss of the PAR-1 antibody binding domain (Figs. 4*b* and 4*c*). This proteolytic process is concentration-dependent (data not shown). These data demonstrate that KLK4 is a potent protease that cleaves PAR-1 at the proper site on live cell surface.

KLK4 activates ERK1/2 and upregulates IL-6 in stromal cells through PAR-1

Downstream signaling of KLK4-mediated PAR-1 activation was then examined in WPMY-1 cells. As shown in Figure 5*a*, KLK4 induced a robust ERK1/2 phosphorylation in WMPY-1 cells in a dose-dependent manner. Similar to thrombin, KLK4 upregulated IL-6 in WPMY-1 cells (Fig. 5*b*). PAR-1 specific effect was confirmed using siRNA against PAR-1. As shown by flow cytometry analysis, the PAR-1 siRNA effectively reduced PAR-1 expression level in WPMY-1 cells by about 75% (Fig. 5*c*, left). Under this condition, PAR-1 siRNA significantly knocked down IL-6 production induced by thrombin (80%) and by KLK4 (60%) (Fig. 5*c*, right). The difference in IL-6 reduction for thrombin *vs.* KLK4 may be due to the presence of PAR-2 (activatable by KLK4 but not by thrombin).[16,18]


a
Non-stimulated
Thrombin-stimulated
b
T-ERK1/2
P-ERK1/2
NS GROα GROβ GROγ IL-6 IL-8 MCP-1
anti-IL-6
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c
IL-6 (pg/ml)
Thrombin 0 0.1 1 10 nM
UO 126
Thrombin
PAR-1 AP
d
GAPDH
T-ERK1/2
Vimentin
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PAR-1 AP
siRNA Control GAPDH ERK1/2
e
PSA
1.0 0.9 1.1 5.1 2.7 5.5 10.2 11.1 10.6
KLK4
1.0 1.1 1.0 2.7 1.5 2.9 4.4 4.9 4.1
GAPDH
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Figure 3.

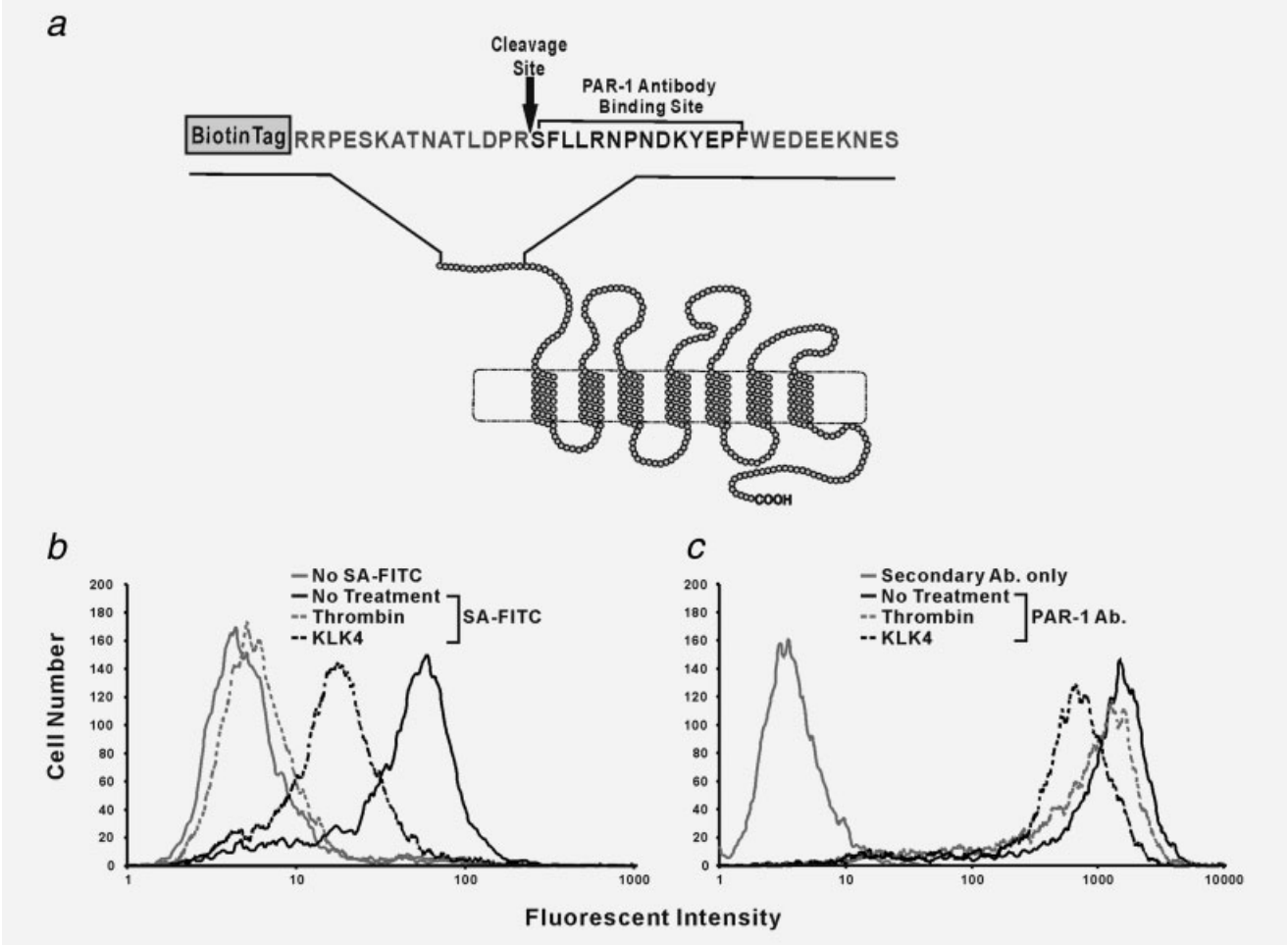
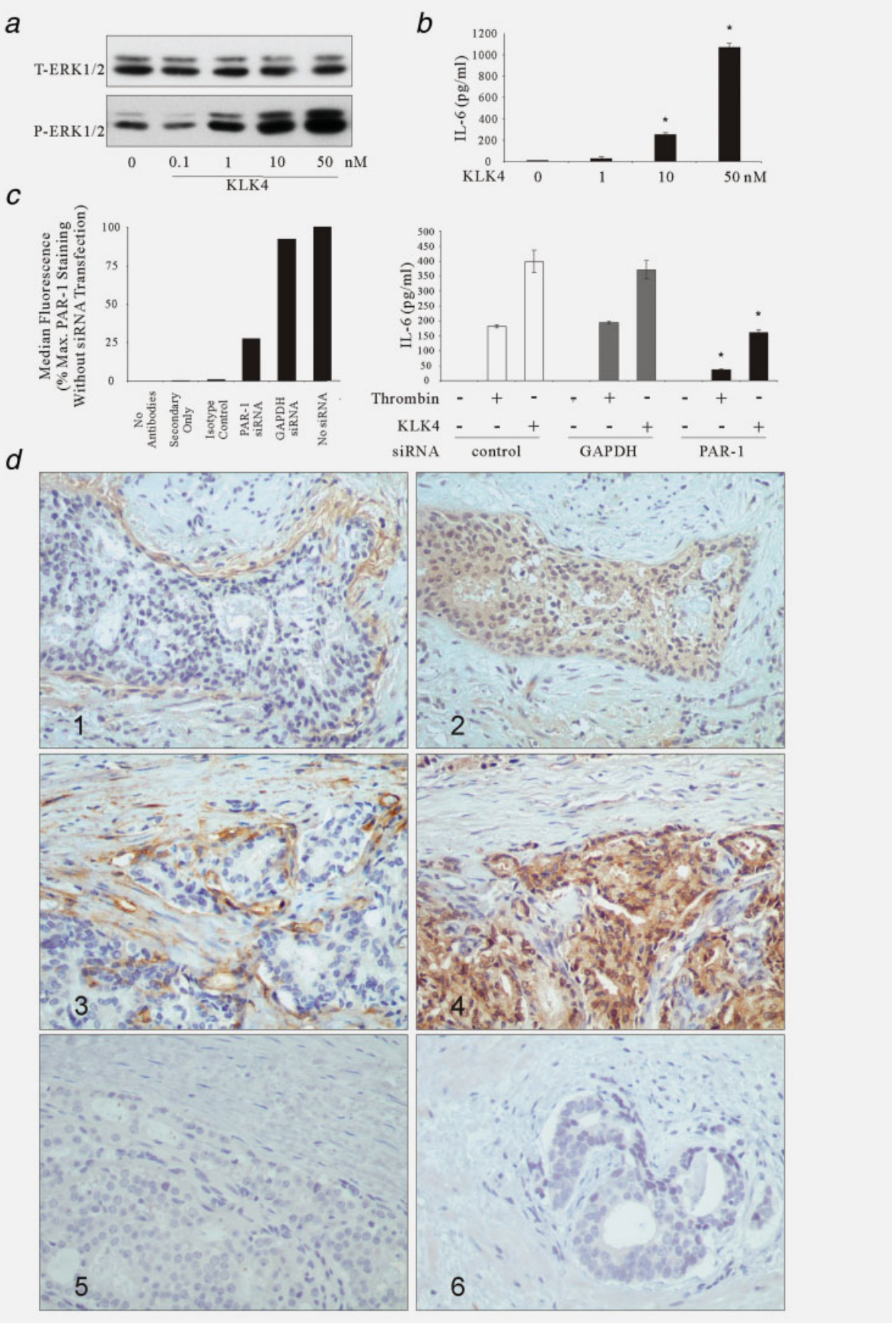


Figure 4. KLK4 cleaves PAR-1 on intact cell surface. (*a*) Diagram of biotinylated PAR-1 construct. Streptavidin-FITC (SA-FITC) detects the N-terminal biotin-tag while the PAR-1 monoclonal antibody (ATAP2) binds to the SFLLRNPNDKYEPF domain, which is located right after the thrombin cleavage site (LDPR/S). (*b,c*) Flow cytometric histograms of thrombin- and KLK4-mediated proteolysis of PAR-1 on CHO-TO cells. Biotinylated PAR-1 on the cell surface was detected with SA-FITC (*b*) or PAR-1 antibody ATAP2 (*c*).

Figure 3. IL-6 is a major cytokine in the thrombin-CM responsible for LNCaP activation. (*a*) Cytokine array analysis of the conditioned medium from WPMY-1 cells. *Top*, Cytokine array map. *Bottom*, Detection of cytokines in the CM from non-stimulated (Left) or thrombin-stimulated (Right) WMPY-1 cells. (*b*) *Left*, LNCaP cells were stimulated with recombinant GROα, GROβ, GROγ, IL-6, IL-8 and MCP-1 (50μg ml^{-1} for 15 min), followed by measurement of ERK1/2 activation. *Right*, CM from thrombin-stimulated WPMY-1 cells (thrombin-CM) was neutralized with anti-IL-6 antibodies (1 μg ml^{-1}) prior to application onto LNCaP cells for 15 min for measurement of ERK1/2 activation. (*c*) ELISA measurement of IL-6 production from PAR-1 induced WPMY-1 cells. *Left*, Dose dependent IL-6 production upon thrombin (0.1–10 nM) stimulation for 24 hr. *Columns*, mean of triplicates; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the non-stimulated cells. *Right*, IL-6 production induced by thrombin (10 nM) or PAR-1 AP (100 μM) in the presence or absence of U0126 (10 μM) for 24 hr. *Columns*, mean of triplicates; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the non-stimulated cells. **$p < 0.05$, compared to the cells exposed to the agonists alone. (*d*) *left*, Knockdown of ERK1/2 expression on WPMY-1 cells by ERK1/2 siRNA. Cells were analyzed by Western blot 48 hr after ERK1/2 siRNA or GAPDH siRNA transfections. Vimentin was used as a loading control. Results are representative of 2 independent experiments. *Right*; Thrombin- or PAR-1 AP-induced IL-6 production in WPMY-1 cells was significantly suppressed by ERK1/2 siRNA treatment but not by GAPDH siRNA. *Columns*, mean of triplicates; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the cells without siRNA treatment. (*e*) Thrombin-CM induces PSA and KLK4 from LNCaP cells *via* IL-6. Thrombin-CM was neutralized without or with anti-IL-6 antibodies (1 μg ml^{-1}) or IgG control (1 μg ml^{-1}) prior to application onto the LNCaP cells for 24 hr followed by Western blot analysis of PSA and KLK4. DHT (10 nM) was used as a positive control. Band intensity was quantified using ImageJ 1.37v (NIH, Bethesda, MD).


a
T-ERK1/2
P-ERK1/2
0 0.1 1 10 50 nM
KLK4
b
IL-6 (pg/ml)
1200
1000
800
600
400
200
0
KLK4 0 1 10 50 nM
c
Median Fluorescence (% Max. PAR-1 Staining Without siRNA Transfection)
100
75
50
25
0
No Antibodies
Secondary Only
Isotype Control
PAR-1 siRNA
GAPDH siRNA
No siRNA
IL-6 (pg/ml)
500
450
400
350
300
250
200
150
100
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Thrombin - + - - + - - + -
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Figure 5.

To seek the evidence of KLK4 and PAR-1 pathological relevance *in vivo*, we examined the expression pattern of PAR-1 and KLK4 in prostate cancer tissue serial sections. PAR-1 was predominantly present in stromal cells surrounding high Gleason grade glands (Fig. 5*d*, 1 and 3), while KLK4 was consistently expressed in the epithelial cancer cells (Fig. 5*d*, 2 and 4). IgG at the same concentration as the corresponding primary antibodies was used as negative control (Fig. 5*d*, 5 and 6). These data demonstrate that PAR-1 and KLK4 are contiguously localized in prostate cancer, and support the hypothesis that they have closely related roles in a paracrine function.

Discussion

We have previously observed that PAR-1 is characteristically localized in peritumoral stromal cells.[26,30] More recently, we and others have found that prostate-specific KLK4 could be a potential activator of PAR-1 and PAR-2.[16,18] During tumor progression, prostate carcinoma cells proliferate and invade through basement membrane into the surrounding stroma, which may expose PAR-1 directly to proteases such as KLK4 produced by the epithelium. Therefore, we tested whether prostate-specific serine proteases may induce the cross-talk between stromal and epithelial cells through a paracrine activation of stromal PAR-1. The present data support a novel mechanism by which activation of PAR-1 in stromal cells by KLK4 upregulates multiple growth factors that in-turn stimulate epithelial cancer growth. We describe this reciprocal process of communication between stromal cells and epithelial cells as a double-paracrine mechanism (Fig. 6).

Increasing evidence suggests that prostate reactive stroma composed primarily of myofibroblasts plays a critical role in regulating epithelial activities through expression of extracellular matrix components, proteases and paracrine growth factors,[42] yet the precise mechanism is not clearly understood. Although it has been suggested that TGF-β1 can induce differentiation of normal fibroblasts to myofibroblasts,[42,43] other mechanisms of reactive stroma generation still remain obscure. PARs are widely distributed receptors that couple to heterotrimeric G proteins which trigger substantial signaling pathways. PAR-1 expression in reactive stromal cells has been observed in breast cancer[44] and prostate cancer,[30] but its cellular function has not been characterized. In the present study, using WPMY-1 as a model for stromal fibroblasts, we show that activation of PAR-1 stimulates ERK1/2 signaling and further induces the secretion of multiple growth factors that promote LNCaP cells growth in the absence of androgen. Consistently, this mechanism was also found in

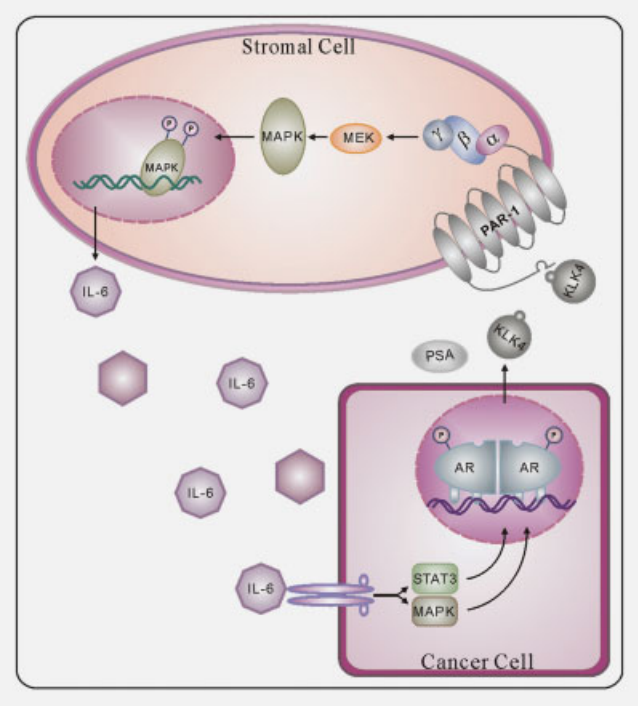

Figure 6. Proposed double-paracrine mechanism of KLK4-initiated and PAR-1-mediated tumor-stroma interaction in prostate cancer. KLK4 initiates the interaction by activating PAR-1 in the stromal cells. PAR-1 signaling leads to the activation of MAPK (ERK1/2) and upregulation of many cytokines, including IL-6. Upon binding to its receptors on cancer cells, IL-6 signaling induces androgen-regulated genes (*PSA* and *KLK4*) in prostate cancer cells by ligand-independent activation of the AR mediated through STAT3 or MAPK pathways.[36] Elevated KLK4 in-turn activates PAR-1 in the stromal cells that may result in a double-paracrine cycle to drive prostate cancer progression.

Figure 5. KLK4 activates ERK1/2 and upregulates IL-6 in stromal cells through PAR-1. (*a*) WPMY-1 cells were stimulated with KLK4 (0.1–50 nM) for 5 min and ERK1/2 activation was measured. (*b*) ELISA measurement of IL-6 production from WPMY-1 cells upon KLK4 (1–10 nM) stimulation for 24 hr. *Columns*, mean of triplicates; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the non-stimulated cells. (*c*) IL-6 response was blocked by PAR-1 siRNA. *Left*, Knockdown of PAR-1 expression on WPMY-1 cells by PAR-1 siRNA. Cells were analyzed using flow cytometry 48 hr after PAR-1 siRNA or GAPDH siRNA transfections. *Columns*, median fluorescence of the sample normalized to nontransfected controls. Results are representative of 2 independent experiments. *Right*, Thrombin- or KLK4-induced IL-6 production in WPMY-1 cells was significantly suppressed by PAR-1 siRNA treatment, while the effect of GAPDH siRNA on IL-6 production was negligible. *Columns*, mean of triplicates; *bars*, SD. Data are representative of 2 separate experiments. *$p < 0.05$, compared to the cells without siRNA treatment. (*d*) Representative immunohistochemical localization of PAR-1 and KLK4 in prostate cancer tissues from 2 patients. Serial sections were stained with PAR-1 antibody (1 and 3) and KLK4 antibody (2 and 4). Mouse IgG (5) or goat IgG (6) was used as negative control. Original magnification: ×200.

primarily cultured human prostate stromal cells (Wang and Takayama, unpublished data). These data suggest that the functional PAR-1 present in stromal cells plays an important role in regulating these growth factors, indicating that PAR-1 may serve as a potential regulator of reactive stroma.

Since PAR-1 plays a critical role in tumor–stromal interactions, identification of the physiological PAR-1 regulator in prostate tumor microenvironment appears to be important. Transcriptional evidence suggests that androgen is involved in regulating PAR-1 mRNA in the early stage of prostate cancer whereas early growth respose-1 (Egr-1) protein may play a central role at the late hormone resistant stage.[45,46] Functionally, human tissue kallikreins have been suggested as potential PAR activators because of their well known trypsin-like activities.[13] By using RNA interference in prostate cancer cells and the PAR-1 knock-out mouse lung fibroblast cell line transfected with either human PAR-1 or PAR-2, we recently demonstrated that KLK4 activates both PAR-1 and PAR-2, whereas KLK2 activates only PAR-2.[18] Another group recently also reported, using Ca^{2+} flux assays, that KLK4 signals through PAR-1 and PAR-2, but not PAR-4.[16] However, evidence of direct PAR-1 cleavage at the activation site by KLK4 was not provided. In the present study, using CHO Tet-on cells expressing biotinylated PAR-1, we demonstrate that KLK4 is capable of cleaving PAR-1 at the proper site on live cell surface. Similar to thrombin, KLK4 further triggers transmembrane signaling and induces IL-6 release by stromal cells *via* PAR-1. Taken together, these results suggest that, as a physiological regulator, KLK4 can initiate the tumor–stroma interactions through activation of PAR-1.

Trypsin-like KLK5, 6 and 14 also have been recently reported as PAR activators,[17] but their biological roles in prostate cancer development are not yet known because they are not highly expressed by the prostate.[47] Besides KLK4, other prostate-secreted proteases that can activate PARs include PAR-2 activators KLK2[18] and TMPRSS2,[48] while KLK3 (PSA), although the most pronounced prostatic protease, is unable to activate PARs due to its chymotrypsin-type activities (Mize and Takayama, unpublished data). Of these KLKs, KLK4 is likely the most significant one in prostate tumor–stromal interactions mediated through PAR-1, because KLK4 is one of the major serine proteases produced by the prostate[49–51] and its ability to activate its substrates (PARs, pro-PSA and pro-urokinase) are much potent than other KLKs (such as KLK2).[15,18] Considering that KLK4 is also able to activate PAR-2,[16,18] which is preferably expressed in prostate cancer cells,[16,30] KLK4 to PAR-2 signaling is likely an autocrine mechanism of epithelial cells. Besides the direct protease-receptor signaling, KLK4 may also contribute to cancer progression by activating other biological substrates.[52] For example, KLK4 may activate the single-chain urokinase plasminogen activator (uPA), which can bind to its cell-surface receptor (uPAR), and then convert plasminogen to plasmin, leading to ECM degradation and the release of growth factors.[13]

How stromal–epithelial interaction leads to prostate cancer toward late stage disease is not clear but may involve androgen-independent AR activation by IL-6. Clinically, serum levels of IL-6 are significantly elevated in patients with hormone refractory prostate cancer.[53] *In vitro*, IL-6 is secreted by androgen-independent DU-145 and PC-3 prostate cancer cells but not the androgen- dependent LNCaP cells, while its receptors are present in all cancer cell lines.[54] This suggests that IL-6 may function as an autocrine growth factor for DU-145 and PC-3 and as a paracrine factor for LNCaP cells. However, how IL-6 is regulated in the prostate tumor microenvironment remains unclear. Here, we show for the first time that KLK4 may function as a physiological regulator of IL-6 production by activating stromal PAR-1. The importance of IL-6 for facilitating prostate cancer growth largely relies on its ability to activate the AR.[39] In particular, the AR N-terminal domain (aa 234-558) appears to be an essential target for IL-6 interaction, which is dependent upon MAPK (ERK1/2) and STAT3 signaling pathways.[55] Following AR activation, IL-6 can subsequently promote prostate cancer cell growth and induce androgen-regulated genes, such as *PSA*.[40,55,56] Therefore, IL-6 has been implicated as an important mediator linking stromal cells or osteoblasts to prostate cancer cells.[33,57,58] Accordingly, our results have demonstrated that IL-6 is a key cytokine in the CM from PAR-1 stimulated stromal cells contributing to ERK1/2 activation, proliferation, and increase of PSA and KLK4 in cancer cells (Fig. 6).

Besides IL-6, stromal PAR-1 activation also significantly upregulates many other cytokines. This finding could be of potential importance as some of these cytokines, including members of the GRO family, IL-8 and MCP-1, are implicated in prostate cancer progression with mechanisms different from the IL-6 pathway. For example, GROα and IL-8 are 2 angiogenic CXC chemokines that can regulate tumor-associated angiogenesis in prostate cancer *in vivo*.[59] Moreover, IL-8 can increase the invasiveness of prostate cancer cells through regulation of matrix metalloproteinase synthesis and secretion[60,61] and promote prostate cancer cell proliferation.[62,63] Both IL-8 and MCP-1 mediate prostate tumor-induced osteoclastogenesis and bone resorption.[64,65] On the other hand, as IL-6 neutralization did not completely block the stromal CM induced mitogenic signaling in LNCaP cells in the present study, these cytokines may also work with IL-6 contributing to the interaction in a synergistic way. Altogether, our data indicate that, upon PAR-1 activation, stromal fibroblasts may serve as a major source of these important cytokines implicated in prostate cancer.

In conclusion, this is the first report to our knowledge that shows the PAR-1 mediated interactions between prostate stromal cells and epithelial cancer cells. As an endogenous PAR-1 activator, epithelium abundant KLK4 initiates the interaction. KLK4-PAR-1 signaling leads to MAPK activation and IL-6 upregulation from stromal cells that further stimulate cancer cells to produce more KLK4 (Fig. 6). Therefore, this double-paracrine interaction may form a cycle that drives carcinoma cell growth progressive to androgen-independent

local invasion and distant metastasis. Future efforts on therapeutics that specifically block the KLK4-PAR-1 pathway in tumor–stroma interactions may prove beneficial in the treatment of prostate cancer.

Acknowledgements

The authors thank Dr. Earl W. Davie (Department of Biochemistry, University of Washington) for critical reading of this manuscript, and Dr. Robert L. Vessella (Department of Urology) and Dr. Lawrence D. True (Department of Pathology) for their support in prostate cancer research.

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