Prostate-Specific Kallikreins-2 and -4 Enhance the Proliferation of DU-145 Prostate Cancer Cells through Protease-Activated Receptors-1 and -2

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Abstract
A major characteristic of prostate cancer is the elevation of serum levels of prostate-specific antigen (hK3) and hK2, which are tumor markers that correlate with advancing stages of disease. Including hK4, these three kallikrein serine proteases are almost exclusively produced by the prostate. Prostate cancer cells have been recently shown to overexpress protease-activated receptors (PAR), which can be potentially activated by kallikreins and can regulate tumor growth. Here, we show that recombinant hK2 and hK4 activate ERK1/2 signaling of DU-145, PC-3, and LNCaP prostate cancer cells, which express both PAR1 and PAR2. These kallikreins also stimulate the proliferation of DU-145 cells. Pretreatment of hK2 and hK4 with the serine protease inhibitor, aprotinin, blocks the responses in DU-145 cells, and small interfering RNA against PAR1 and PAR2 also inhibits ERK1/2 signaling. To determine which PAR is activated by hK2 and hK4, a cell line that expresses a single PAR, a PAR1 knockout mouse lung fibroblast cell line transfected with PAR1 (KOLF-PAR1) or PAR2 (KOLF-PAR2) was used. hK4 activates both PAR1 and PAR2, whereas hK2 activates PAR2. hK4 generates more phosphorylated ERK1/2 than hK2. These data indicate that prostatic kallikreins (hK2 and hK4) directly stimulate prostate cancer cell proliferation through PAR1 and/or PAR2 and may be potentially important targets for future drug therapy for prostate cancer. (Mol Cancer Res 2008;6(6):1043–51)

Introduction
Although the use of the tumor marker prostate-specific antigen (PSA) has had an effect on the management of prostate cancer, which causes more than 30,000 deaths annually in the United States (1), the biological role of this enzyme in cancer progression is unclear (2). PSA is a member of the tissue kallikrein (hK, KLK) family of genes, located on chromosome 19q13.4 (2, 3). There are 15 members, and 3 (hK2, hK3, and hK4) are almost exclusively produced by the epithelial cells of the prostate and are considered prostate-specific (2, 3). The suggested function of PSA in cancer includes direct or indirect activation of mitogenic proteins (e.g., latent TGFβ, PTHrp, and IGFBP), and degradation of extracellular matrix to facilitate cancer spread (2, 3). hK2 is very similar to PSA, with an 80% amino acid sequence identity, and also has potential functions including activation of the cancer-mediator urokinase-type plasminogen activator and degradation of extracellular matrix (2, 3). hK4, most recently discovered through differential expression analysis of androgen-regulated prostate cancer cells, is less similar to PSA (40% amino acid identity; ref. 4), but has much more potent enzyme activity than hK2 or PSA (2, 5). hK4’s suggested functions include cleavage of IGFBP and tooth enamel matrix proteins as well as the stimulation of osteoblast migration and PC-3/DU-145 proliferation through unknown mechanisms (2, 6). Although kallikreins can proteolytically cleave substrates with biological properties, whether or how kallikreins can directly stimulate cancer cells is mostly unknown. This article explores the novel process of prostate cancer (LNCaP, DU-145, PC-3 cells) activation by hK2 and hK4 through protease-activated receptors (PAR).

One of the well-recognized mechanisms whereby serine proteases such as kallikreins (7) can directly stimulate cells is through PARs (8-10). PARs are G protein–coupled receptors that are activated when their amino-terminal extracellular domain is cleaved by a serine protease to “unmask” a new NH2 terminus which serves as a “tethered ligand” (8, 9). The ligand then binds to the second extracellular loop of the G protein–coupled receptor, resulting in intracellular events that vary with the particular cell type. The four PAR members include PAR1 (thrombin receptor; ref. 11), which is primarily known for the activation of platelets, and PAR2 (12), which seems to be involved in the regulation of inflammation and wound-healing processes (9). Although the physiologic role of human PAR3 (13) is unknown, PAR4 (14, 15) seems to be responsible for the slow and sustained activation of platelets by thrombin (9). The specific in vitro activation of each PAR has been shown by the use of PAR-specific activation peptides (AP) based on each “tethered ligand” (9) that binds to and activates only one PAR receptor. More recently, PAR1 knockout mouse lung fibroblast (KOLF) cells (lacking PAR1 and PAR2) have been transfected to express individual human PAR1 or PAR2 to help test PAR-specific agonist activities (16).

Evidence for the potential role of PARs in cancer development includes PAR-overexpression and PAR-dependent cancer behavior (9, 10). In many cancers, increased PAR...
expression occurs in both primary and metastatic tissues (17) compared with the normal organ. Cancer types that overexpress PAR1 and PAR2 include breast, ovary, stomach, lung, liver, and thyroid (17). Furthermore, various cancers exhibit PAR-dependent growth (18), invasion (19, 20), and metastatic behavior (21). In prostate cancer, PAR1 is clearly overexpressed in advanced cancer (22, 23). Up-regulated PAR1 in malignant prostate tissue is an independent predictor of subsequent biochemical recurrence, whereas PAR2 is also overexpressed in cancer compared with normal prostate glands (22). PAR1 expression in bone-derived prostate cancer cells was originally discovered on cDNA analysis (24), and subsequently, both PAR1 and PAR2 agonists were shown to stimulate RhoA-dependent cytoskeletal changes in the androgen-sensitive LNCaP prostate cancer cell line (25). In addition, PAR activation of various prostate cancer cell lines results in the expression of potential cancer mediators (25-32), increased proliferation (33), and enhanced migration (34). Therefore, PARs are likely to be quite important in prostate cancer development.

Results

Prostate Cancer Cell Lines Express PAR1 and PAR2

We previously showed that the LNCaP prostate cancer cells express PARs and are stimulated by PAR agonists (25). Here, we show the DU-145 and PC-3 cell lines, which are more aggressive compared with the LNCaP cells and represents more advanced prostate cancer (35), also express PAR1 and PAR2, as shown by flow cytometry (Fig. 1).

hK2 and hK4 Stimulate Prostate Cancer Cells (DU-145, PC-3, and LNCaP) through PAR1 and/or PAR2 (ERK1/2 signaling)

To test whether hK2 and hK4 can directly stimulate PARs on prostate cancer cells, the effects of these kallikreins on DU-145, PC-3, and LNCaP cells were examined. The cells were activated in serum-free medium by agonists for 3 minutes then measured for phosphorylation of ERK1/2 by Western blot (Fig. 2). Both of the PAR1 and PAR2 agonists (thrombin or AP-1 and trypsin or AP-2, respectively) generate robust ERK1/2-signaling in the DU-145 and PC-3 cells (Fig. 2A and B). ERK1/2-signaling in LNCaP cells (Fig. 2C) results only with AP-2 and with trypsin (PAR2), and not with AP-1 or thrombin (PAR1). When stimulated with either hK2 or hK4, the DU-145 and PC-3 cells also have stronger response (A and B), compared with the LNCaP cells (C). The potency of hK4 is at least 3-fold greater than hK2, in both DU-145 and PC-3 cells. The LNCaP cells have some response to hK4 and none to hK2. Taken together, the main difference between the three cell lines tested is that the DU-145 and PC-3 cells have ERK1/2 signaling response to both PAR1 and PAR2, whereas LNCaP only responds to PAR2 stimulation.

Endogenous Expression of hK2 and hK4 in DU-145, PC-3, and LNCaP Cell Lines

To detect the endogenous mRNA levels of hK2 and hK4 in all three cell lines, semiquantitative reverse transcription-PCR was done (Fig. 3). Androgen-dependent LNCaP cells express endogenous hK2 and hK4. However, for androgen-independent DU-145 and PC-3 cells, endogenous mRNA levels of hK2 and hK4 are undetectable. Our data is consistent with the previous reports (4, 35, 36), which show that hK2 and hK4 expression is androgen-dependent.

ERK1/2 Activation and Enhanced Proliferation by PAR Agonists

Because DU-145 cells responded well to all agonists, we focused on this cell line for the further characterization of kallikrein activation of PAR1 and/or PAR2. ERK1/2-signaling is important for G protein–coupled receptor–mediated cell fate (37), consequently, the time course of ERK1/2 activation by PAR stimulation was studied. Both AP-1 (Fig. 4A) and thrombin (Fig. 4B) stimulation triggers a prompt increase in ERK1/2 phosphorylation (peaks at 3-5 minutes) which decreases over the next 8 hours. ERK1/2-mediated proliferation was then evaluated...
by tritiated thymidine incorporation (Fig. 4C). All of the stimuli significantly enhance DU-145 cell proliferation, with the maximal effect achieved by hK4 (1.7-fold over nonstimulated) and AP-1 (1.7-fold), compared with hK2 (1.3-fold), thrombin (1.3-fold), trypsin (1.6-fold), and AP-2 (1.5-fold).

Activation of PAR1 or PAR2 by hK2 or hK4 is Blocked by the Serine Protease Inhibitor, Aprotinin

In order to confirm that the active enzyme form of the kallikreins stimulated the DU-145 cells directly, the effect of aprotinin was assessed (Fig. 5). The serine protease agonists were preincubated with a 5-fold molar excess of aprotinin for 3 minutes prior to use in stimulating the cells. In control cells, aprotinin has no effect on ERK1/2-signaling (Fig. 5A), but in stimulated cells, it completely blocks the effects of hK2 and hK4. Aprotinin also completely abrogates the proliferative response to the serine proteases (Fig. 5B).

Activation of PAR1 or PAR2 by hK4 is Blocked by Small Interfering RNA against PAR1 and/or PAR2

For confirmation of PAR-specific stimulation of the DU-145 cells by hK4, the cells were transfected with either PAR1 small interfering RNA (siRNA) and/or PAR2 siRNA (Fig. 6). The control siRNA against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) decreases the expression of GAPDH on Western blot without significantly altering PAR1 or PAR2 activation (Fig. 6A, top). The PAR1 and PAR2 siRNA individually decrease ERK1/2-signaling by hK4 (Fig. 6A, bottom), whereas the combination of PAR1 and PAR2 siRNA more completely inhibits the ERK1/2-signaling (bottom lane, far right). The specificity of PAR1 and PAR2 siRNA against their respective PAR was also confirmed using AP-1 and AP-2, respectively.

Flow cytometry analysis (Fig. 6B), shows a reduction in PAR1 or PAR2 levels 48 hours after transfection with their respective PAR1 or PAR2 siRNA. The PAR1 siRNA reduces PAR1 expression by 50% with no reduction in PAR2 levels. The PAR2 siRNA reduced PAR2 expression by 40% with no reduction in PAR1 levels.

hK2 Activates PAR2 whereas hK4 Activates both PAR1 and PAR2 (ERK1/2-Signaling and Proliferation) in KOLF Cells

To confirm whether the kallikreins activate either or both PAR1 and PAR2, we analyzed the effects of hK2 and hK4 on KOLF cells stably transfected with human PAR1 or PAR2. Cells were stimulated by the PAR agonists and kallikreins in the same fashion as the DU-145 cells above. The wild-type KOLF cells (KOLF-WT) that lack PAR1 and PAR2 expression do not generate any PAR-specific ERK1/2-signaling by any of the agonists including the hK2 or hK4, but have intact signaling with epidermal growth factor stimulation (Fig. 7A). The PAR-expressing KOLF cells generate ERK1/2-signaling when stimulated by either their respective agonists (thrombin, AP-1 for KOLF-PAR1; Fig. 7B); trypsin, AP-2 for KOLF-PAR2 (Fig. 7C). When these cells are stimulated with kallikreins, hK4...
activates PAR1 whereas hK2 does not activate PAR1. Both hK2 and hK4 activate KOLF-PAR2 cells. Furthermore, hK4 is much more potent in generating phosphorylated ERK1/2 (~3-fold) than hK2 (Fig. 7C). The optimum dose required to stimulate the cells by hK2 and hK4 were previously determined to be 50 nmol/L for each (data not shown). The level of activation by hK4 was very similar to the activation achieved by the control proteases, thrombin and trypsin for PAR1 and PAR2, respectively. Furthermore, hK4 seems to be just as potent in the activation of PAR1 as PAR2. Results suggest that hK4 is more likely to be the physiologic activator of PAR1 and PAR2 than hK2 in the prostate. In addition, the results indicate that there is a simultaneous activation of both PAR1 and PAR2 by hK4.

In order to determine the downstream effects of hK2 and hK4 on the transfected KOLF cells, we analyzed the effects of these kallikreins on cell proliferation. The control KOLF-WT cells do not respond to any of the agonists but do have intact proliferation with epidermal growth factor stimulation (Fig. 8A). AP-1 (2.8-fold) and hK4 (2.5-fold) have the maximum effect on proliferation of KOLF-PAR1 cells (Fig. 8B), followed by thrombin (2.1-fold). hK2 has essentially no effect on the KOLF-PAR1 cells. For KOLF-PAR2 cells (Fig. 8C), trypsin (3.7-fold) has the maximum effect, followed by AP-2 (2.3-fold), hK4 (2-fold), and hK2 (1.6-fold). The data therefore confirm that hK4 activates both PAR1 and PAR2, whereas hK2 primarily activates PAR2 and is significantly less potent than hK4.

Discussion

Although PSA was first identified in human seminal plasma in 1969, it was not until its subsequent use as a prostate cancer tumor marker in the mid-1980s that its clinical value was realized (2). The reported functions of kallikreins are generally divided into (a) regulating growth by cleavage and release of peptide mediators and (b) degradation of extracellular matrix proteins (2). Although the physiologic activators of kallikreins are not known, pro-PSA is activated in vitro by hK2, hK4, and...
hK15 (5, 38-41). It is perhaps unusual that the human prostate gland produces so many of the kallikreins. Only the pancreas seems to express more kallikreins in substantial amounts (2). These prostate-associated kallikreins are hormone-regulated and as such are likely involved in the maturation and development of the adult prostate. All of these kallikreins are elevated in prostate cancer; however, little is known about their biological importance in the development of prostate cancer.

Here, our focus was on hK2 and hK4, which are the only trypsin-type kallikreins whose expression is almost exclusive to the prostate.

Because PARs are G protein–coupled receptors that are activated by serine proteases, the prostatic kallikreins are potentially important activators of these receptors and mediators involved in cancer. PARs have been shown to have a role in the metastatic behavior of various cancers including breast and colon cancers (9, 10). When activated, the downstream effects in cancer cells include proliferation, invasion, and release of angiogenic factors (9, 10). Many of the receptors, including PAR1 and PAR2, are also up-regulated in many cancers (17). We recently showed that the up-regulation of PAR1 in prostate cancer tissue specimens was associated with subsequent biochemical recurrence of disease as shown by an elevation of serum PSA levels (22). Therefore, it is plausible that both the increase in the levels of kallikreins (hK2, hK3, and hK4) and the simultaneous elevation of PARs may be biologically significant in the genesis of prostate cancer. Because the prostate produces multiple kallikreins and PARs, in vitro analysis of specific PAR activation by a particular kallikrein would be necessary to decipher their relative role(s) in cellular proliferation.

Recently, some kallikreins (hK5, hK6, and hK14; ref. 7) besides hK2 and hK4 have been shown to activate PARs in vitro, but the potential role(s) of this interaction in the cancer development and organ-specific nature is not yet known. In this study, both the prostate-specific kallikreins, hK2 and hK4, were shown to activate PAR(s) in prostate cancer cells (DU-145, PC-3, and LNCaP). Furthermore, which PAR is/are activated in particular was evaluated with KOLF cells that express either PAR1 or PAR2. We found that whereas hK4 can activate PAR1 and PAR2, hK2 can only activate PAR2. When compared with each other, hK4 is much more potent than hK2 in activating...
PAR2. This is similar to the ability of hK4 to activate pro-PSA and pro-urokinase much better than hK2 (5). The optimal concentrations of hK2 and hK4 were 50 nmol/L (data not shown), which were consistent with significant levels found in the prostate (2, 42). Tissue expression levels of serine protease(s) may therefore be very important in the biological activation of a given PAR and worth further investigation in the future.

The activation of PAR1 and/or PAR2 by hK2 and hK4 results in not only the stimulation of ERK1/2 signaling, but also leads to enhanced subsequent cellular proliferation. The protease action and the PAR-specific effects were confirmed by the use of trypsin-inhibitor (aprotinin) to block the kallikrein activity and siRNA directed against PAR1 and/or PAR2, respectively. Of note is that the GAPDH siRNA treatment results in a mild reduction of total ERK1/2 and phosphorylated ERK1/2 (Fig. 6). This effect likely represents some biological function of GAPDH in these cells. The higher potency of hK4 compared with hK2 and their difference in activation of PARs (KOLF data) described above could be due to substrate-specificity difference based on the 11 amino acid insertion in front of the catalytic aspartate in hK2 (“kallikrein loop”; ref. 2). Accordingly, hK4 is more likely to be the physiologic activator of PAR1 and PAR2 in the prostate than hK2.

Of the two prostate-specific trypsin-type kallikreins, hK4 seems to have other divergent functions depending on the
cellular compartment in which it is expressed (2, 3). Xi and colleagues reported that hK4 may be localized in the nucleus where it acts as a proliferative factor (43, 44). They recently showed with cell cycle-specific oligonucleotide array analysis that hK4 can up-regulate proliferative proteins such as cyclin B1 and E2F1 (43). On the other hand, several other investigators have shown that hK4 is a secreted protein (45, 46) and therefore has the capability to activate cell surface receptors such as PARs. Whether the overexpression of hK4 leads to prostate cancer development is unknown but is an intriguing possibility.

Although prostate cancer cells have been previously shown to express functional PARs, whether prostate-associated kallikreins can directly stimulate prostate cancer cells through PARs have not been previously reported. This study shows hK2/hK4-mediated PAR activation in three separate prostate cancer cell lines (DU-145, PC-3, and LNCaP). Both the DU-145 and the PC-3 cells, which were derived from advanced prostate cancer, have been extensively used to study the mechanisms of prostate cancer progression (35). The LNCaP cell line reflects a less-advanced cancer that retains androgen dependence (35). Here, we show that all three cell lines produce high levels of both PAR1 and PAR2, and that these receptors are functional in generating ERK1/2 signaling when activated by hK2 and/or hK4. Interestingly, LNCaP differs from the other two cell lines because PAR1 agonists do not stimulate ERK1/2 signaling in the LNCaP. This finding occurs despite the fact that PAR1 agonists activate RhoA signaling in these same cells. To our knowledge, this is the first time that PAR signaling has been shown to differ between different prostate cancer cells. This difference may also be relevant to prostate cancer progression. For example, PAR1-mediated ERK1/2 signaling in the more aggressive cells (DU-145 and PC-3) may be an acquired trait that was not present in the more indolent state (LNCaP). The yet unanswered question is whether there is autocrine activation of PARs by prostatic kallikreins. Because the LNCaP cells do express hK2 and hK4 as shown here, such a possibility does exist. However, the evaluation of autocrine activation by kallikreins is complex and beyond the scope of this article.

It would require sophisticated kinetic studies with PAR substrates to measure active enzyme(s) released by the cells and the use of enzyme-specific substrates and inhibitors that could differentiate serine protease activities (e.g., hepsin, TMPRSS2, and other serine proteases of the prostate). Because kallikreins are released in their precursor proenzyme forms, this could also hamper autocrine studies. Autocatalytic activation of pro-hK2 has been previously shown (38, 39, 41, 47), whereas the native activation of pro-hK4 has not been reported. Future work would be necessary to first show whether inhibiting kallikrein(s) would prevent ambient activation/internalization of PAR(s).

In conclusion, this is the first report to our knowledge that shows prostatic kallikrein-mediated activation of PARs in prostate cancer cells. Future therapeutic efforts to treat patients with advanced prostate cancer may therefore benefit from targeting prostate-specific kallikreins and/or PARs because the kallikrein-PAR mechanisms may be important in prostate cancer progression.

Materials and Methods

Materials

Human α-thrombin (specific activity 2,983 NIH units/mg; 8,949 NIH units/mL) was purchased from Enzyme Research Laboratories. Trypsin from bovine pancreas (10,000 BAEE units/mg), human epidermal growth factor, protease inhibitor cocktail, PBS, and saponin were obtained from Sigma Chemical Company. Amidated PAR-activating peptides (PAR1 = AP-1 NH2-TFLLRN-NH2; PAR2 = AP-2 NH2-SLIGKV-NH2) were from Anaspec, Inc. ECL Plus Western blot detection kit was from GE Healthcare Bio-Sciences, Corp. Polyvinylidene difluoride was from Millipore. Antibodies against total and phosphorylated (Thr202/Tyr204) ERK1/2 were from Cell Signaling Technology. Antibodies against PAR receptors, HRP-conjugated secondary antibodies for Western blotting, and FITC-conjugated secondary antibodies for fluorescence-activated cell sorting were from Santa Cruz Biotechnology. RPMI 1640 and DMEM were from Mediatech, Inc. Fetal bovine serum was from Gemini Bio-products. Penicillin and streptomycin were from Invitrogen. Hygromycin B was from Calbiochem.

Cell Lines and Culture

DU-145, PC-3, and LNCaP from American Type Culture Collection were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL). KOLF-WT, human PAR1-transfected KOLF cells (KOLF-PAR1), and human PAR2-transfected KOLF cells (KOLF-PAR2; ref. 48) were kindly provided by Dr. Shaun R. Coughlin and Dr. Eric Camerer (Cardiovascular Research Institute, University of California, San Francisco, CA). KOLF cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL). KOLF-PAR1 and KOLF-PAR2 cells were maintained in 200 μg/mL of hygromycin B.

Recombinant hK2 and hK4

Human recombinant kallikreins, hK2 (41) and hK4 (5), were purified and characterized as previously described.

Semiquantitative Reverse Transcription-PCR

DU-145, PC-3, and LNCaP cells were grown to 80% confluence and total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from the RNA samples by using Moloney murine leukemia virus reverse transcriptase and random decamers (Ambion). For semiquantitative PCR analysis, GAPDH primers were used as the internal control, and hK2 and hK4 primers were used for specific amplification. For GAPDH, primers used were F, 5′-GTGGGAGATCTCGAGAATTAC-3′; and R, 5′-TCAAGGCTTCTGGGATTC-3′. For hK2, primers used were F, 5′-AGAATGGTACCATACACG-3′; and R, 5′-GAGATCTGTACACCTTGTTTGA-3′. For hK4, primers were used as F, 5′-AGAATGCTACCATACACG-3′; and R, 5′-GGATGTGCTACACCTTGTTTGA-3′. For hK2, primers were used as F, 5′-GGGAGATCTCGAGAATTAC-3′; and R, 5′-GGATGTGCTACACCTTGTTTGA-3′.

FACS Analysis

DU-145 or PC-3 cells grown to 80% confluence were analyzed as previously described (25). Primary monoclonal
antibodies against PAR1 (ATAP2) or PAR2 (SAM11) were used at a 1:100 dilution and the FITC-conjugated antimouse IgG was used at a 1:400 dilution. The washed cells were analyzed on a Becton Dickinson FACScan flow cytometer.

**ERK1/2 Signaling Assay**

At 80% confluence, the cells were washed and switched to serum-free medium for 24 h. The cells were washed thrice with PBS and activated in serum-free medium for the indicated times with the following agonists: thrombin (10 nmol/L final concentration), trypsin (5 nmol/L), hK2 and hK4 (50 nmol/L), and PAR-activating peptides (15 μmol/L). The activated cells were then washed thrice with cold TBS [25 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L MgCl₂, 0.37 mmol/L Na₃HPO₄, 0.9 mmol/L CaCl₂] and lysed with cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.2), 1% Triton X-100, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl₂] containing 30 mmol/L of DNasel and protease inhibitor cocktail (18 mmol/L AEBSF, 8 μmol/L aprotinin, 0.36 mmol/L bestatin, 0.15 mmol/L pepstatin A, 0.14 mmol/L E-64, and 0.21 mmol/L leupeptin). Cell lysates were kept on ice for 10 min, centrifuged (13,000 × g for 3 min at 4°C), then analyzed by Western blot to detect the phosphorylated forms of ERK1/2.

**Treatment with siRNA against PAR1 and PAR2**

Transfection of DU-145 cells with PAR1 and PAR2 SMARTpool (75 nmol/L) siRNAs (Dharmacon) or Silencer GAPDH siRNA (Ambion) with DharmaFECT1 (0.5 μL) was done with a modification of the double-transfection method (49). The cells were transfected for 5 h, the growth medium replaced, and the cells were treated with a second transfection after 24 h. The cells were reincubated (24 h) then shifted to serum-free medium prior to the ERK signaling assay.

**Proliferation Assay**

Cell proliferation was analyzed by thymidine incorporation. Subconfluent cells detached with EDTA (0.5 mmol/L) were plated on 24-well culture dishes at 50,000 cells per well (DU-145 cells) or 25,000 cells per well (KOLF cells) for 24 h. The cells were washed thrice with PBS and switched to serum-free medium for 24 h. The cells were then activated in fresh serum-free medium with the following agonists: thrombin (10 nmol/L final concentration), trypsin (5 nmol/L), hK2 and hK4 (10 nmol/L), and PAR-activating peptides (15 μmol/L). After 24 h, 1 μCi of [³H]thymidine (GE Biosciences) was added for 5 h. Unincorporated [³H]thymidine was removed by washing thrice with cold PBS. DNA was precipitated overnight by the addition of 5% TCA. Precipitates were dissolved in 0.5 mol/L of NaOH, 0.5% SDS, diluted with Opti-fluor scintillation cocktail (Packard) and counted using a Packard Tri-carb 1500 liquid scintillation counter.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Earl W. Davie and members of the Davie lab, Paul H. Lange, and Robert L. Vessella for their continued support of our prostate cancer research. We also thank Shaun R. Coughlin and Eric Camerer for their advice regarding PARs and KOLF cells, and for providing the KOLF cells.

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