Altered Protease–Activated Receptor-1 Expression and Signaling in a Malignant Pleural Mesothelioma Cell Line, NCI-H28, with Homozygous Deletion of the β-Catenin Gene

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Abstract
Protease activated receptors (PARs) are G-protein coupled receptors that are activated by an unique proteolytic mechanism. These receptors play crucial roles in hemostasis and thrombosis but also in inflammation and vascular development. PARs have also been implicated in tumor progression, invasion and metastasis. In this study, we investigated expression and signaling of PAR1 in nonmalignant pleural mesothelial (Met-5A) and malignant pleural mesothelioma (NCI-H28) cells. We found that the expression level of PAR1 was markedly higher in NCI-H28 cells compared to Met-5A and human primary mesothelial cells. Other three malignant pleural mesothelioma cell lines, i.e. REN, Ist-Mes2, and Mero-14, did not show any significant PAR1 over-expression compared to Met-5A cell line. Thrombin and PAR1 activating peptides enhanced Met-5A and NCI-H28 cell proliferation but in NCI-H28 cells higher thrombin concentrations were required to obtain the same proliferation increase. Similarly, thrombin caused extracellular signal-regulated kinase 1/2 activation in both cell lines but NCI-H28 cells responded at higher agonist concentrations. We also determined that PAR1 signaling through Gq and G12/13 proteins is severely altered in NCI-H28 cells compared to Met-5A cells. On the contrary, PAR1 signaling through Gq proteins was persistently maintained in NCI-H28 cells. Furthermore, we demonstrated a reduction of cell surface PAR1 expression in NCI-H28 and malignant pleural mesothelioma REN cells. Thus, our results provide evidences for dysfunctional PAR1 signaling in NCI-H28 cells together with reduced plasma membrane localization. The role of PAR1 in mesothelioma progression is just emerging and our observations can promote further investigations focused on this G-protein coupled receptor.

Introduction
Malignant mesothelioma (MM) is a relatively rare but highly aggressive neoplasm arising from mesothelial cells on the serosal surfaces of the pleural, peritoneal and pericardial cavities. Asbestos fiber exposure is widely accepted as the main cause with approximately 80% of cases being directly attributed to occupational exposure [1]. Although asbestos exposure has a pivotal role in initiating both cellular and molecular events which lead to MM development other factors such as genetic and epigenetic alterations contribute to its pathogenesis [1]. Several growth factors and their target receptors have been implicated in the oncogenesis, progression and resistance to therapy of MM [1]. In addition, the chemokine CXL12 and its target receptor CXCR4 which belongs to the large family of seven-transmembrane G-protein coupled receptors (GPCRs), have been found to be highly expressed in malignant pleural mesothelioma (MPM) cell lines and tumor tissues suggesting they can be involved in tumor progression and survival [2].

Numerous evidences link aberrant GPCR expression and activation to several types of human malignancies [3,4]. Among GPCRs, PARs are a subset which have a unique mechanism of activation. In fact, they are activated enzymatically through proteolysis by enzymes of the serine protease family [5]. The proteolytic cleavage occurs at specific sites within their N-terminal region, thereby exposing novel N-termini, and the ‘tethered ligand’ then folds back onto the extracellular loop II of the receptor, resulting in activation. There are four PARs encoded by distinct genes in the mammalian genome. The prototype of this GPCR subfamily is PAR1 which transmits cellular response to thrombin [6,7]. The receptor subfamily also includes PAR2 which is activated by trypsin, and two other thrombin-activated receptors, PAR3 and PAR4 [8–10]. Other proteases besides trypsin for PAR2 and thrombin and trypsin for PAR3 and PAR4...
can activate these receptors [11]. Additionally, synthetic peptides that mimic the first six amino acids of the newly formed N-terminus can act as soluble ligands in the absence of receptor proteolysis. Activated PAR1 couples to multiple heterotrimeric G-protein subtypes including Gq, G12 and G13 [11,12].

PARs have multiple roles in many physiological and pathological events involving different tissues and organs such as the cardiovascular, musculoskeletal, gastrointestinal, respiratory and central nervous system [13]. Coagulant proteases and PARs have been implicated in several types of malignant cancer. PAR1 is over-expressed in aggressive melanoma, colon cancer, prostate cancer, and invasive breast cancer [14–17], promoting tumor cell invasion and epithelial cell malignancy [17–20]. In addition, several proteases, which can activate PAR1, have been identified in tumors including tissue-derived trypsins, members of the coagulation cascade and matrix metalloprotease-1 [18,21]. Finally, a recent study has shown that MPM cell lines that express tissue factor and PAR1 but not PAR2 are able to generate large tumors in nude mouse thoracic cavities [22].

In the present study, we analyzed PAR1 expression levels, signaling and mitogenic effects in immortalized nonmalignant pleural mesothelial (Met-5A) and MPM cells (NCI-H28). In this MPM cell line, a homozygous deletion of the β-catenin gene (CTNNB1) has been demonstrated while thrombomodulin, a natural anticoagulant, appears to be silenced by an epigenetic mechanism [23,24]. Therefore, we were interested to study PAR1 expression and signaling in this cell line and correlate our findings to known genetic and epigenetic alterations. Our work indicates that the expression levels of both PAR1 mRNA and protein are increased in NCI-H28 cells compared to those found in Met-5A and primary human mesothelial cells. In addition, the increased PAR1 expression appears to be an unique feature of the NCI-H28 cell line since in other three MPM cell lines, i.e. REN, Mero-14 and Ist-Mes2, PAR1 levels are not significantly different from that found in Met-5A cells. Perhaps more important, PAR1 signaling to downstream effectors is dysfunctional as the signaling pathway through Gq is the only one that is fully maintained while G12/13 and Gq35 pathways are reduced. Furthermore, the mitogenic effect triggered by PAR1 activation is modified in NCI-H28 cells as compared to Met-5A cells. We also show that in this MPM cell line, cell surface PAR1 expression is reduced and the receptor mainly localizes in the intracellular compartment. The intracellular retention of PAR1 is likely responsible of the altered signaling.

Materials and Methods

Materials

Penicillin, streptomycin, hydrocortisone, cAMP, 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone, protease inhibitor cocktail, isoproterenol and secondary antibodies were products of Sigma-Aldrich Inc. (St. Louis, MO, USA). [3H]-cAMP (specific activity 31.0 Ci/mmol) and enhanced chemiluminescence substrate (Western lightning Plus-ECL) were from PerkinElmer Inc. (Waltham, MA, USA). The human microvascular endothelial cell (HMEC-1) line [25] was a generous gift of E.W. Ades (Centers for Disease Control, Atlanta, GA, USA) while NCI-H28 and Met-5A cells were purchased from LGC Standards s.r.l. (Middlesex, UK). REN mesothelioma cells [26,27] were a generous gift of L. Moro (University of Piemonte Orientale “A. Avogadro”, Italy) while Mero-14 [28] and Ist-Mes2 [29] mesothelioma cells were kindly donated by Istituto Nazionale per la Ricerca sul Cancro (IST) - Genova (Italy). Mero-14, Ist-Mes2 and REN cells were verified for their identity by analyzing 10 to 18 genetic markers. Human adult primary mesothelial cells and their growth medium (MSO-1) were purchased from Zen-Bio, Inc (Research Triangle Park, NC, USA). Medium 199, MCDB-131 medium, RPMI-1640, DMEM, fetal bovine serum (FBS), trypsin-EDTA, epidermal growth factor (EGF), L-glutamine, human recombinant insulin, nitrocellulose membrane, Lipopectamine 2000 transfection reagent, Flu-3 acetoxy methylester (Fluo-3 AM), phoronic acid, Alexa Fluor 488- and Alexa Fluor 568-labeled goat anti-mouse IgG and anti-rabbit IgG antibodies were purchased from Life Technologies Corporation (Carlsbad, CA, USA). Halt phosphatase inhibitor cocktail and 2,2’-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (1 step) were from Thermo Scientific (Waltham, MA, USA). WST-1 was a product of La Roche (Basel, Switzerland). RhoA activation assay kit was obtained from Cytoskeleton, Inc. (Denver, CO, USA). The PAR1 antagonist, SCH 27979 and the selective PAR1-activating peptide (PAR1-AP) (TFLLR-NH2) were products of Tocris Bioscience (Bristol, UK). The non-selective PAR1-AP (SFLRR-NH2) was synthesized in Dr. A.M. D’Ursi’s laboratory (Department of Pharmacy, University of Salerno, Fisciano, Italy) using a conventional solid-phase strategy based on the Fmoc/t-Bu protection chemistry as previously described [30]. Human α-thrombin (high activity, ≥ 2,800 NIH U/mg protein) was a product of Calbiochem (EMD Millipore Biosciences, Billerica, MA, USA). The RNeasy Mini Kit and SYBR Green PCR Kit were purchased from Qiagen GmbH (Hilden, Germany). The Rev Transcription Kit was a product of New England Biolabs (Ipswich, MA, USA). A polyclonal anti-PAR1 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) while a monoclonal anti-PAR1 antibody was from Abnova (Taipei City, Taiwan). A rabbit polyclonal anti-PAR1 antibody generated against the N-terminal sequence YEPFWEDEEKNESGLTVEYG was a generous gift of Dr. J. Trejo (Department of Pharmacology, University of California, San Diego, CA, USA) [31]. Polyclonal anti-β-catenin, anti-caveolin-1, anti-phospho-p44/42 MAPK (extracellular signal-regulated kinase 1/2; ERK1/2), and anti-p44/42 MAPK (ERK1/2) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). A monoclonal anti-β-catenin antibody was from BD Biosciences (San Jose, CA, USA). A monoclonal anti-β-actin antibody was purchased from EMD Millipore Biosciences (Billericia, MA, USA). A vector containing the human β-catenin cDNA (pCMV6XL5-β-catenin-1), the pCMV6XL5 vector, a small interfering RNA (siRNA) directed against β-catenin, and a scrambled non-targeting siRNA (control siRNA) were purchased from OriGene (Rockville, MD, USA). Other agents and reagents were from standard commercial sources and were of the highest grade available.

Cell culture

Met-5A cells were grown in Medium 199 supplemented with 10% FBS, 1% penicillin/streptomycin (100 units/ml/100 µg/ml), hydrocortisone (400 nM), EGF (3.3 nM) and human recombinant insulin (870 nM). NCI-H28 and REN cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (100 units/ml/100 µg/ml). Ist-Mes2 and Mero-14 cells were grown DMEM medium containing 4.5 g/ml glucose and 3.97 mM L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin (100 units/ml/100 µg/ml). Cells were normally propagated in their own growth media except before experiments they were plated in RPMI-1640 medium. Primary mesothelial cells were cultured in MSO-1 medium (Medium 199 supplemented with FBS, EGF, penicillin, streptomycin, and amphotericin B) according to manufacturer’s instructions. HMEC-1 cells were cultured as previously described [32]. All cells were cultured at 37°C and 5% CO2 in humidified atmosphere.
Real time RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) and tested for integrity by gel electrophoresis. mRNA was reverse transcribed to cDNA using a specific Rev Transcription Kit (New England BioLabs). Real time SYBR Green polymerase chain reaction (PCR) for PAR1 was performed using forward primer: 5'-TGCTTCAGTCTGTGCGG-3' and reverse primer: 5'-CTCCATCAATAAAAGCAGTCCTCT-3'. The relative expression of PAR1, with β-actin as the reference gene, was determined using the MiniOpticon Real-Time PCR Detection System (BioRad Laboratories, Inc., Hercules, CA, USA). Data are presented as expression ratios normalized to β-actin.

Western blot analysis

Human primary mesothelial cells grown in MSO-1 medium, Met-5A, NCI-H28, REN, Mero-14, and IstMes2 cells cultured in complete RPMI-1640 medium until confluence were washed with ice-cold PBS (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4, 157 mM NaCl and 2.7 mM KCl) and lysed in modified RIPA buffer (PBS, pH 7.4, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 10 μl/ml protease inhibitor cocktail). Lysed cells were centrifuged at 14,000 g at 4°C for 45 min and supernatant was collected. To measure the protein content, the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used with bovine serum albumin (BSA) as standard. Solubilized proteins (30 μg) were separated by 12% SDS-PAGE and transferred onto nitrocellulose. Immunoblots were carried out using a standard method as previously described [32,33]. The immunoblot signal was visualized by using enhanced chemiluminescence substrate detection system (Western Lightning Plus-ECL). The chemiluminescent images were acquired by LAS4010 (GE Healthcare Life-Sciences, Pittsburgh, PA, USA). Intensity of immunoreactive bands was measured by densitometric scanning using Image Quant TL 1D, Version 7.0 (GE Healthcare Life-Sciences, Pittsburgh, PA, USA). Nitrocellulose membrane probed with anti-PAR1 antibodies was subsequently stripped and reprobed with the anti-β-actin antibody.

ERK1/2 activity was determined from 18 h serum and growth factor starved cells plated at 3 x 10⁵ density in 6-well dishes. After stimulation with different thrombin concentrations for 5 min, cells were lysed in modified TBS (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Igepal, 10% glycerol, 1% protease and phosphatase inhibitor cocktails) and processed as described above. Activated ERK1/2 was detected by immunoblotting with anti-phospho-p44/42 MAPK (ERK1/2) antibody. Membranes were stripped and reprobed with anti-p44/42 MAPK (ERK1/2) antibody.

Immunocytochemistry

NCI-H28 and Met-5A cells were seeded at 3 x 10⁴ cells per well in chamber slide (BD Biosciences, San Jose, CA, USA). Twenty-four hours later, cells were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer, washed three times with PBS, rinsed, and...
blocked for 45 min with PBS containing 0.1% Triton-X 100 and 1% BSA. After washing, cells were incubated with mouse monoclonal anti-PAR1 (1:100), mouse monoclonal anti-β-catenin (1:500) or rabbit polyclonal anti-β-catenin (1:1000) and rabbit polyclonal anti-caveolin-1 (1:400) primary antibodies diluted in PBS containing 0.03% Triton-X 100 and 1% BSA for 18 h at 4°C. Double labeling studies were carried out as follow: anti-PAR1 and anti-caveolin-1; anti-PAR1 and rabbit polyclonal anti-β-catenin; mouse monoclonal anti-β-catenin and anti-caveolin-1. After washing, to visualize single staining, cells were incubated with Alexa Fluor 488- and Alexa Fluor 568-labeled goat anti-mouse (1:400) or anti-rabbit (1:400) antibodies for 2 hour at room temperature. Then slides were covered with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Confocal images were obtained with a Leica TCS-SP5 confocal microscope, using a 40×oil objective with 1.45 NA and a recommended pinhole size of less than 1.0 micrometer. The images were processed with PhotoshopCS3 software. To evaluate fluorescence colocalization, the images were also analyzed using the freely available ImageJ program [34].

**Cell proliferation assay**

Met-5A and NCI-H28 cells were plated at 3×10^3 cells/well in clear 96-well dishes and allowed to adhere overnight. Then cells were serum and growth factor starved for 12 hours and stimulated with and without agonists for 72 hours. After that, 10 μl of WST-1 mixture was added to each well, mixed gently for one min and cells incubated for additionally 2 hours at 37°C. Finally, the formazan dye was quantified by measuring the absorbance of each sample against background as blank with a Wallac 1420 multilabel counter microplate reader (PerkinElmer, Inc., Boston, MA, USA) at a wavelength of 450 nm.

**[Ca^{2+}]_i measurement**

PAR-induced increase of [Ca^{2+}]_i, was assessed by measuring fluorescence variations after agonist stimulation of cells loaded with Fluo-3 AM using a Wallac 1420 multilabel counter microplate reader (PerkinElmer, Inc., Boston, MA, USA), as previously described [32]. Cells were seeded in black/clear bottom 96-well assay plates at a density of 2×10^5 cells/well (HMEC-1) or 1.5×10^5 cells/well (Met-5A and NCI-H28) in complete growth media. After attachment, cells were starved in serum and growth factor free media containing BSA for 3 h at 37°C. Before starting the assay, cells were washed twice with loading buffer (20 mM
probenecid and then incubated in 100 mM containing 25 mM mannose, 1 mg/ml BSA and 2.5 mM 6-mercaptoethanol. The amount of PAR 1 on plasma membrane was quantified by ELISA essentially as described by Paing et al. Assays were initiated by the addition of 1 μM selective PAR1-AP using a G-LISA RhoA activation assay kit (San Diego, CA, USA).}

**Measurement of intracellular cAMP**

Intracellular cAMP levels were measured using a competitive protein binding method as previously described [32]. Met-5A and NCI-H28 cells [4×10^4] well were plated in 24-well dishes and allowed to grow for 24 h. Thereafter, cells were incubated for 15 min in serum and growth factor free media containing 20 μM 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone and then exposed to different thrombin or selective PAR1-AP concentrations in the presence and absence of 100 nM SCH 79797 for 15 min. Assays were initiated by the addition of 1 μM isoproterenol.

**Cell surface ELISA**

Detection of endogenous PAR1 expressed on the cell surface was quantified by ELISA essentially as described by Paing et al. [35]. Met-5A and NCI-H28 cells were plated in 24-well dishes at 6×10^4 cell/well and grown overnight. Assay media was RPMI-1640 supplemented with 1 mg/ml BSA and 1% penicillin/streptomycin. Cells were washed with media and incubated on ice for 30 min. Afterwards, cells were washed and incubated with 5 μM SCH 79797 for 30 min and then treated with 10 nM thrombin for 10 min at 37°C. Cells were then washed with PBS and fixed with 4% paraformaldehyde on ice for 10 min. After fixation, cells were washed with PBS and incubated with primary antibody for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugate goat anti-rabbit secondary antibody for 1 h at room temperature. After washing, cells were incubated with horseradish peroxidase substrate 1 step 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt at room temperature. An aliquot was removed from each well and optical density was determined at 405 nm using a Wallac 1420 multilabel counter microplate reader.

**Data analysis**

Data analysis was performed by the computer program GraphPad Prism Version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Values represent the means ± S.E.M. of at least three independent experiments. The statistical significance of value differences was evaluated by one-way ANOVA followed by Bonferroni’s multiple comparison test using GraphPad Prism Version 4.0 for Windows. The Pearson’s correlation coefficient was used as statistic for quantifying fluorescence colocalization in confocal images.

**Results**

PARs and their potential activating proteases are frequently over-expressed in human tumor tissues, including prostate cancer, invasive breast cancer, colon cancer, and malignant melanoma [14–20]. Lee et al. [36] have shown that PAR2 is present in human pleural tissues where it plays a role in pleural inflammatory responses while in primary cultures of human peritoneal mesothelial cells the expression of PAR1 has been reported [37]. In addition, the expression of PAR1 has been revealed in 3 MPM cell lines by western blot analysis but these cell lines do not express PAR2 [22]. Therefore, we decided to investigate expression and signaling of PAR1 in human pleural mesothelial and MPM cells to evaluate the possible role of this receptor in mesothelioma cell proliferation. For this work we utilized the MPM cell line, NCI-H28, which does not express CXCR4 and the nonmalignant pleural mesothelial cell line, Met-5A, was used as a control [2]. In this MPM cell line, apart from a homozygous deletion of the β-catenin gene (CTNNB1) a down-regulation of thrombomodulin expression by an epigenetic mechanism has been described [23,24]. The expression of thrombomodulin, a glycosylated transmembrane protein which binds with high affinity to thrombin inhibiting its enzymatic activity and accelerating protein C activation, is lower in MPM tissue than in normal mesothelium [24]. In addition, low or no expression of thrombomodulin in various cancers has been associated with poor prognosis [38–40].
PAR1 is over-expressed in NCI-H28 cells

To verify whether PAR1 mRNA level was different in malignant NCI-H28 cells compared to nonmalignant Met-5A cells, real time RT-PCR was performed using RNA extracted from these cells. In NCI-H28 cells, PAR1 mRNA level was significantly increased compared to Met-5A cells (Figure 1.A). Immunoblot analysis showed a 48 kDa band corresponding to PAR1 in lysates of Met-5A, NCI-H28 and other three MPM (Ist-Mes2, REN and Mero-14) cell lines while two close bands were detectable in immunoblot of human primary mesothelial cell lysates (Figure 1.C). The appearance of two bands was not a surprise since human PAR1 contains multiple glycosylation consensus sites and several studies have shown the detection of 40 to 100 kDa bands on immunoblots [41–43]. However, the PAR1 protein expression was lower in primary mesothelial cells than in Met-5A cells (Figure 1.B and 1.C). In NCI-H28 cells, the protein expression level was significantly increased compared to primary mesothelial and Met-5A cells (Figure 1.B and 1.C). In the other MPM cell lines, PAR1 protein levels were essentially similar to that found in Met-5A cells. Therefore, the increased PAR1 expression is an unique feature of NCI-H28 cell line. Overall, these findings suggest that the increased expression of PAR1 in NCI-H28 cells results from increased gene transcription although enhanced PAR1 mRNA and/or PAR1 protein stability can also be involved. We also examined PAR2 mRNA and protein levels in Met-5A and NCI-H28 cells (data not shown). Real time RT-PCR and western blot analysis demonstrated PAR2 expression levels were similar in both cell lines.

Figure 4. PAR1 agonist-induced Gq but not Gi signaling is impaired in NCI-H28 cells. A, thrombin-induced intracellular Ca2+ mobilization in HMEC-1, Met-5A, and NCI-H28 cells. B, selective-PAR1-AP-induced intracellular Ca2+ mobilization in Met-5A and NCI-H28 cells. Serum and growth factor starved cells were loaded with fluo-3AM to measure [Ca2+]i, variations as indicated by changes in fluorescence intensity. Fluorescence was recorded before agonist addition (F0) and then every 3 seconds after thrombin (10 nM) or PAR1-AP (10 μM) addition for another 120 seconds. Data shown are mean ± SEM of a single experiment done in triplicate. Experiments were repeated two additional times with similar results. The results are reported as relative fluorescence (RF = F/F0 where F0 is basal fluorescence and F is fluorescence recorded after cell stimulation with the agonist). C, inhibition of isoproterenol stimulated cAMP production in Met-5A and NCI-H28 cells by different concentrations of thrombin in the presence and absence of 100 nM SCH 79797. D, no effect of the selective PAR1-AP on isoproterenol stimulated cAMP production in Met-5A and NCI-H28 cells. Serum and growth factor starved cells were exposed to different agonist concentrations. Assays were initiated by the addition of 1 μM isoproterenol. Production of cAMP was measured using a competition binding assay which includes the bovine adrenal cAMP binding protein and [3H]cAMP. Data shown are mean ± SEM of three independent experiments performed in triplicate. The differences between thrombin- and thrombin plus SCH 79797-treated cells were significant (**P<0.01, ***P<0.001) by one-way ANOVA followed by Bonferroni’s multiple comparison test (n = 3).

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PAR1 agonists enhance Met-5A and NCI-H28 cell proliferation

Next, we examined whether in NCI-H28 cells, PAR1 was functionally active by evaluating thrombin- or PAR1-APs-induced cell proliferation. Met-5A and NCI-H28 cells were incubated with various thrombin or PAR1-AP concentrations for 72 h. In Figure 2.A, the proliferative responses induced by thrombin stimulation are reported. Both Met-5A and NCI-H28 cells showed significant increases of cell proliferation at 72 h (Figure 2.A). However, the pattern of the proliferative response was quite different in NCI-H28 cells compared to that of Met-5A cells. As an example, in Met-5A the proliferative response was maximal at 1 nM thrombin with a progressive decrease up to 50 nM while in NCI-H28 cells the maximal response was reached at 50 nM (Figure 2.A).

The non-selective PAR1-AP, SFLLRN-NH2, was less effective than thrombin in stimulating Met-5A and NCI-H28 cell proliferation (Figure 2.B). A 24–28% increase of cell proliferation was reached at 10 and 100 μM SFLLRN-NH2 in Met-5A and NCI-H28 cells, respectively (Figure 2.B). The selective PAR1-AP,

Figure 5. PAR1 agonist-induced G12/13 signaling is impaired in NCI-H28 cells. A, relative levels of RhoA activation in response to thrombin in Met-5A and NCI-H28 cells. B, relative levels of RhoA activation in response to the selective PAR1-AP in Met-5A and NCI-H28 cells. RhoA activation was measured in serum and growth factor starved cells using the RhoA G-LISA kit from Cytoskeleton. Data shown are mean ± SEM of three independent experiments performed in triplicate. The differences in RhoA activation between Ctrl (vehicle treated Met-5A or NCI-H28 cells) and agonist-treated cells were significant (*P<0.05, **P<0.01, ***P<0.001) by one-way ANOVA followed by Bonferroni’s multiple comparison test (n=3).

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TFLLR-NH₂ was less efficacious in stimulating cell proliferation than SFLLRN-NH₂ but a concentration of 100 μM caused a 20% increase of NCI-H28 cell proliferation (Figure 2.C). These results highlight that PAR1-APs do not behave exactly as thrombin in stimulating cell proliferation.

Reduced cell surface PAR₁ expression in NCI-H28 cells

Since NCI-H28 cells respond with proliferation at higher thrombin concentrations even though they express increased PAR₁ levels (see Figure 1), we questioned whether the receptor is properly localized on cell surface in this cell line. Therefore, we compared the amount of cell surface PAR₁ in Met-5A, NCI-H28 and REN cells using an ELISA assay. Interestingly, NCI-H28 cells showed significantly less cell surface PAR₁ expression than Met-5A cells (Figure 3). REN cells, which express β-catenin as indicated by immunoblot analysis (data not shown), also showed a reduced cell surface receptor expression compared to Met-5A cells (Figure 3). Overall, these findings provide evidences of an altered cell surface distribution of PAR₁ in two MPM cells lines showing different levels of total receptor expression.

Dysfunctional PAR₁ signaling in NCI-H28 cells

To further explore PAR₁ ability of signaling in the NCI-H28 cell line, receptor-activated G<sub>i</sub>, G<sub>q</sub>, and G<sub>12/13</sub> signaling pathways...
were examined. First, we investigated PAR1-activated Gq signaling by analyzing intracellular Ca\textsuperscript{2+} mobilization after cell stimulation with either thrombin or the selective PAR1-AP. As indicated by relative fluorescence increase, both thrombin (10 nM) and PAR1-AP (10 \textmu M) induced rapid and transient increase of [Ca\textsuperscript{2+}]\textit{i} in Met-5A as well as in HMEC-1 as previously reported (Figure 4.A and 4.B) [32]. In contrast, thrombin- or PAR1-AP-stimulation of NCI-H28 cells resulted in a reduced increase of [Ca\textsuperscript{2+}]\textit{i} (Figure 4.A and 4.B). Given the sharply contrasting results, we examined both cell lines for the expression levels of some
components of the G₄ signaling pathway by immunoblot analysis (Figure S1). Whereas PLC-β₁ was expressed at similar levels in both cell lines, the amount of G₅ₙ was apparently greater in NCI-H28 than Met-5A cells (Figure S1).

To explore the functional integrity of Gi signaling pathway, we analyzed thrombin- and PAR₁-AP-induced inhibition of isoproterenol stimulated cAMP accumulation in both Met-5A and NCI-H28 cells. In Met-5A cells, 10 pM to 1 nM thrombin inhibited isoproterenol stimulated cAMP production in a concentration dependent manner reaching 50% inhibition at 1 nM (Figure 4.C). However, at higher thrombin concentrations (1 nM to 100 nM) the inhibitory effect was progressively diminished. In the presence of SCH 79797, the inhibitory effect of thrombin was reduced indicating that PAR₁ mediates the effect. In NCI-H28 cells, thrombin inhibited cAMP in a concentration dependent manner reaching 50% and maximal inhibition (approximately 70%) at 1 nM and 100 nM, respectively (Figure 4.C). In the presence of SCH 79797, the inhibition curve was upwards shifted and the maximal inhibition at 100 nM was only 42% indicating that the inhibitory effect of cAMP accumulation is partially mediated by PAR₁. Various concentrations of the selective PAR₁-AP did not cause any inhibition of isoproterenol stimulated cAMP production in both Met-5A and NCI-H28 cells (Figure 4.D) demonstrating the functional selectivity of this peptide agonist.

Next, we examined PAR₁-activated G₁₂/₁₃ signaling by measuring RhoA activation after cell stimulation with either thrombin or PAR₁-AP. In Met-5A cells, 10 nM thrombin induced a significant 2.5-fold increase of RhoA activation while in NCI-H28 cells the increase was just 1.2-fold (Figure 5.A). The selective PAR₁-AP (10 μM) was less effective in stimulating RhoA activation than thrombin in Met-5A cells but it still caused a significant increase (Figure 5.B). Similarly to thrombin, PAR₁-AP induced a modest increase of RhoA activation in NCI-H28 cells (Figure 5.B). We also examined the expression levels of G₁₂, G₁₃, and RhoA in both cell lines by immunoblot analysis (Figure S1). Our results indicate G₁₂ and RhoA expression levels were similar in Met-5A and NCI-H28 cells while G₁₃ expression was significantly increased in NCI-H28 cells compared to Met-5A cells (Figure S1).

To further investigate distinctions in signaling, we examined thrombin induced ERK1/2 activation, an important mitogenic signaling cascade, in Met-5A and NCI-H28 cells. Thrombin (10 nM) caused a rapid increase of phosphorylated-ERK1/2 (pERK1/2) which reached a maximum level at 5 min and persisted up to 30 min in both cell lines (data not shown). Using a single time point (5 min) we examined the effect of various thrombin concentrations ranging from 0.01 to 100 nM and found that a maximal response was induced by 0.1 nM thrombin in Met-5A cells while higher thrombin concentrations reduced pERK1/2

![Figure 9. Neither β-catenin rescue nor deletion modify cell surface PAR₁ expression.](image-url)

NCI-H28 cells were transiently transfected with plasmide vector containing CTNNB1 or empty vector (Ctrl) while Met-5A cells were transfected with nonspecific (Ctrl) or specific β-catenin siRNA as described in Materials and Methods. A, relative expression levels of β-catenin. Transfected cells were lysed and total cell proteins were analysed by immunoblot using an anti-β-catenin antibody. Then membranes were reprobed with an anti-β-actin antibody. Data are expressed as arbitrary unit (fold variation over Ctrl) after normalization by β-actin. Data shown are mean ± SEM of three independent experiments. The differences of β-catenin relative levels between Ctrl s and cell transfected with the recombinant vector or specific siRNA were significant (*P<0.05) by one-way ANOVA followed by Bonferroni’s multiple comparison test (n=3). B, a representative immunoblot. C, cell surface PAR₁ expression measured by ELISA assay. Antibody binding to fixed transfected cells was detected by horseradish peroxidase-conjugated secondary antibody. Data represent the mean ± SEM of three independent experiments performed in triplicate. The differences in cell surface PAR₁ expression between Ctrl s and cell transfected with the recombinant vector or specific siRNA were significant (**P<0.001) by one-way ANOVA followed by Bonferroni’s multiple comparison test (n=3).

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Prevalent intracellular PAR1 localization in NCI-H28 cells

In human umbilical vein endothelial cells, it has been reported that β-catenin greatly facilitates recruitment of caveolin-1 to VE-cadherin/catenin complex at cell junctions [44]. Additionally, several lines of evidence indicate that caveolae are relevant for GPCRs/G proteins signaling including that driven by PAR1 [45,46]. As NCI-H28 cells have a homozygous deletion of the β-catenin gene we questioned whether the lack of this protein could reduce cell membrane recruitment of both caveolin-1 and PAR1. Therefore, we analyzed β-catenin, caveolin-1 and PAR1 localization in Met-5A and NCI-H28 cells by immunocytochemistry (Figure 7 and 8). In Met-5A cells, both β-catenin and caveolin-1 were localized on the plasma membrane including at some cell junctions and PAR1 also showed a prevalent but not exclusive localization on the plasma membrane (Figure 7). In contrast, in NCI-H28 cells there was no β-catenin staining, and caveolin-1 and PAR1 were mainly localized in the cytoplasm (Figure 7). In Met-5A cells, double labeling studies suggested β-catenin and caveolin-1 closely localized at cell junctions. In addition, both intracellular and plasma membrane PAR1 apparently colocalized with caveolin-1 (Figure 8). In NCI-H28 cells, the intracellular PAR1 was also in close proximity to caveolin-1 as suggested by the yellow stain (Figure 8). A quantification of PAR1/caveolin-1 colocalization using Pearson’s correlation coefficient (PCC) indicated a good degree of correlation in both Met-5A (PCC = 0.77 ± 0.05; n = 6) and NCI-H28 cells (PCC = 0.84 ± 0.03; n = 6).

Neither β-catenin rescue nor deletion affect cell surface PAR1 expression

In order to test our hypothesis that β-catenin is required for proper cell surface PAR1 localization, we transiently transfected NCI-H28 cells with a plasmid vector containing human β-catenin cDNA and silenced β-catenin expression in Met-5A cells using a specific siRNA. Immunoblot analysis indicated that in NCI-H28 cells transfected with the recombinant vector, β-catenin was expressed at high levels (Figure 9A and 9B) compared to the expression level in cells transfected with the empty vector. On the other hand, we also obtained a consistent reduction of β-catenin expression in Met-5A cells transfected with the β-catenin siRNA as compared to cells treated with a nonspecific scrambled siRNA (Figure 9A and 9B). However, in ELISA assays β-catenin transfected NCI-H28 cells did not show any increase of cell surface PAR1 expression while silenced Met-5A cells had no significant decrease of cell surface receptor as compared to control cells (Figure 9C). Using immunofluorescence microscopy, we were also unable to detect any important change of PAR1 localization in β-catenin transfected and silenced cells as compared to respective controls (data not shown). All together, our findings indicate that the lack of β-catenin is not responsible for reduced cell surface PAR1 localization.

Discussion

Coagulant proteases and PARs have been implicated in several types of malignant tumors. Indeed, a well-documented link between hyperactivation of the coagulation cascade and tumor progression exists. The pro-coagulant activity mediated by the action of coagulant proteases such as thrombin can contribute to the malignant phenotype both directly, by stimulating tumor cell proliferation, and indirectly through the development of tumor-associated thromboemboli [47]. Among cancer patients, those with MMP are very susceptible to thromboembolic complications [48]. In addition, Keshava et al. [22] have shown that MMP cell lines, which express tissue factor and PAR1, generate large tumors in mouse thoracic cavity thus indicating that activation of PAR1 promotes MMP cell growth.

To this end we investigated whether a correlation exists between PAR1 expression and cell proliferation using a MMP cell line (NCI-H28) and a nonmalignant pleural mesothelial cell line (Met-5A). In the NCI-H28 cell line, thrombomodulin, a transmembrane glycoprotein that controls thrombin-mediated proteolysis, is silenced by an epigenetic mechanism [24]. We found that the proliferative response of NCI-H28 cells to various thrombin concentrations was quite different from that obtained with the nonmalignant pleural mesothelial cell line. Whereas in NCI-H28 cells, thrombin-induced proliferation increased in a concentration dependent fashion, in Met-5A cells thrombin induced the maximal effect at 1 μM and then at higher concentrations the stimulatory effect progressively decreased. The proliferative response of NCI-H28 cells increased without reaching any growth steady state as expected when cells lose contact inhibition, a typical characteristic of cancer cells. The diverse response can result as consequence of reduced cell surface localization of PAR1 (Figure 3) in NCI-H28 cells even though the total receptor amount is increased. However, we do not feel to exclude that the lack of thrombomodulin in NCI-H28 cells [24] affects PAR1 growth signaling.

The non-selective PAR1-AP, SFLLRN-NH2, enhanced proliferation of both nonmalignant pleural mesothelial and MMP cells in a concentration-dependent fashion [13]. However, the proliferative response was slightly less marked than that observed with thrombin suggesting that either thrombin is also acting through other receptors or PAR1 activation by proteolytic cleavage elicits a cellular response which is not completely identical to that induced by a “free” synthetic peptide agonist. Backhart et al. [49] have reported that distinct cellular responses can be evoked by thrombin versus synthetic peptide agonists. In addition, McLaughlin et al. [50] have demonstrated that thrombin-activated PAR1 preferentially couples to G12/13 proteins while PAR1-APs favor activation of Gα signaling leading to [Ca2+]i increase. The modest enhance of cell proliferation induced by the selective PAR1-AP suggests that PAR1 may also contribute to thrombin- and SFLLRN-NH2-stimulated functional response in both cell lines. Although thrombin is not able to cleave and activate PAR2, thrombin-cleaved PAR1 can transactivate PAR2 in human umbilical vein endothelial cells [51]. Indeed, as mentioned before, we were able to detect similar levels of PAR2 expression in Met-5A and NCI-H28 cells (data not shown).

When PAR1-mediated activation of signaling pathways was examined, we immediately noticed that Gq and G12/13 Signaling was compromised in NCI-H28 cells. In this MMP cell line, the only signaling pathway which was fully activated by thrombin-cleaved PAR1 is through Gq proteins leading to inhibition of adenyl cyclase. Indeed, thrombin inhibited cAMP production in a concentration-dependent fashion in NCI-H28 cells while in Met-5A cells it showed a biphasic effect. Simultaneous activation of different G proteins with release of a plethora of Gβγ subunits which are able to activate some isoforms of adenyl cyclase can be responsible for the biphasic shape of the curve [52]. It is interesting to note that the selective PAR1-AP did not cause any major inhibition of cAMP accumulation. These findings are in agreement with thrombin and PAR1-AP displaying functional selectivity at PAR1 as reported by McLaughlin et al. [50].
Decreased Gq and G12/13 signaling with the prevalence of Gi signaling can explain the altered proliferative response to thrombin in NCI-H28 cells. Indeed, PAR1-mediated activation of ERK1/2 occurs through both Gq and Gi signaling with consequent activation of mitogenesis [53]. When we examined thrombin-induced ERK1/2 activation we found that lower thrombin concentrations were able to activate ERK1/2 in Met-5A than in NCI-H28 cells. This finding supports the role of Gi signaling in mediating thrombin-induced ERK1/2 activation in Met-5A. Persistent PAR1 signaling as consequence of altered receptor trafficking has been reported in metastatic breast carcinoma cells leading to enhanced cellular invasion [20]. We might speculate that altered PAR1 signaling can also impact MPM cell invasiveness.

Compartmentalization of PARs and G proteins in plasma membrane lipid raft microdomains such as caveolae can confer PAR/G protein selectivity [21]. Russo et al. [46] have shown the critical role of caveole in activated protein C (APC) activation of PAR1 selective signaling in endothelial cells. Furthermore, some studies concerning other GPCRs have demonstrated that caveolin-1 is required to prolong Gq signaling and inhibit receptor coupling to G(q) proteins [54,55]. In thrombin-stimulated endothelial cells, caveolin-1 opens cell junction by targeting catenins [44]. The recruitment of caveolin-1 at cell junctions is greatly facilitated by the presence of β-catenin in the cadherin/catenin complex. In NCI-H28 cells, a homozygous deletion of the β-catenin gene (CTNNB1) has been demonstrated suggesting that in these cells caveolin-1 is not completely associated to the plasma membrane [21]. Our immune fluorescence experiments show that in NCI-H28 cells caveolin-1 is partially retained in the cytoplasm while in Met-5A cells it is prevalently localized to the plasma membrane. In Met-5A cells, PAR1 is distributed in both plasma membrane and intracellular compartments and double immunolabeling studies suggest its proximity to caveolin-1. In NCI-H28 cells, PAR1 is mostly retained in the intracellular compartment. Of note, PAR1 and caveolin-1 appear to colocalize in both cell lines as suggested by PCC values. The intracellular retention of the receptor is confirmed by ELISA showing a consistent reduction of cell surface PAR1 in NCI-H28 cells compared to Met-5A cells. However, we do not know whether in NCI-H28 cells the increased intracellular receptor distribution is due to altered cell surface recruitment or enhanced-internalization of activated receptor. Of note, REN cells, another MPM cell line, which express similar PAR1 levels than Met-5A cells, also show a reduction of cell surface PAR1 by ELISA assay (see Figure 3). This aggressive MPM cell line does not express thrombomodulin as the NCI-H28 cell line and expresses high levels of tissue factor and very little amount of endothelial cell protein C receptor [22]. Thus, these evidences suggest that the observed reduction of cell surface PAR1 expression in these MPM cell lines can result as consequence of activated-receptor internalization. In order to exclude a role of β-catenin in recruiting PAR1 to the plasma membrane, we performed both rescue and deletion experiments and evaluated cell surface receptor expression by ELISA. However, our findings suggest that β-catenin expression is not required for cell surface PAR1 localization in both NCI-H28 and Met-5A cells. Since the NCI-H28 cell line is only one among other MPM cell lines examined, which shows a highly significant increase of PAR1 expression compared to Met-5A and human primary mesothelial cells, we may speculate that β-catenin indirectly modulates PAR1 expression at transcriptional level.

In summary, we have demonstrated that PAR1 is highly over-expressed in a MPM cell line, NCI-H28, while other three MPM cell lines show similar or slightly increased expression levels than a mesothelial cell line (Met-5A) and human primary mesothelial cells. Thrombin promotes Met-5A and NCI-H28 cells proliferation through activation of PAR1. In NCI-H28 cells, PAR1 although over-expressed, is defective in cell surface localization and signaling through Gq and G12/13 pathways. Cell surface PAR1 expression is also reduced in MPM REN cells, thus suggesting receptor activation and internalization by cell produced proteases in both cell lines. Further studies are needed to investigate the role of cell surface or secreted proteases in inducing PAR1 activation and stimulation of MPM growth.

Supporting Information

Figure S1 Expression of Gq subunits, RhoA, PLCβ1, and caveolin-1 in Met-5A and NCI-H28 cells. Cells were lysed and protein solubilized as described under Materials and Methods. Proteins were then separated by SDS-PAGE and transferred onto nitrocellulose. Specific anti-Gqα, -Gqβ, -Gqγ, -RhoA, -PLCβ1, and -caveolin-1 antibodies were used to detect each protein. Nitrocellulose membranes were subsequently stripped and re-probed with an anti-β-actin antibody. The intensity of the immunoreactive bands was quantified by densitometric scanning. A, relative intensity of immunoreactive bands. Data are expressed as arbitrary unit (fold increase over Ctrl, Met-5A) after normalization by β-actin. Data shown are mean ± SEM of three independent experiments. The differences in protein expression between Met-5A and NCI-H28 cells were significant (p<0.05) by one-way ANOVA followed by Bonferroni’s multiple comparison test (n = 3). B, a representative immunoblot. Polyclonal anti-Gqα antibodies were obtained from ABCAM (Cambridge, UK) while monoclonal anti-RhoA and polyclonal anti-PLCβ1 antibodies were from EMD Millipore Biosciences (Billerica, MA) and Thermo Fisher Scientific (Waltham, MA) respectively. (TIF)

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Author Contributions

Conceived and designed the experiments: MRM AL. Performed the experiments: AF VD YDV FC IP AC CG. Analyzed the data: AF VD. Contributed the reagents/materials/analysis tools: LG AMD. Wrote the paper: MRM.

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