PAR-type thrombin receptors in renal carcinoma cells: PAR_1-mediated EGFR activation promotes cell migration

SEBASTIAN BERGMANN[1], KERSTIN JUNKER[2], PETER HENKLEIN[3], MORLEY D. HOLLENBERG[4], UTZ SETTMACHER[1] and ROLAND KAUFMANN[1]

Departments of [1]General and Visceral Surgery, and [2]Urology, University Hospital, Friedrich Schiller University Jena, Erlanger Allee 101, D-07747 Jena; [3]Institute of Biochemistry, Charité, Humboldt University of Berlin, Monbijoustr. 2, D-10117 Berlin, Germany; [4]Canadian Institutes of Health Research Proteinases and Inflammation Network, Endocrine-Diabetes, Smooth Muscle and Mucosal Inflammation Groups, Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Canada

Received October 20, 2005; Accepted December 12, 2005

Abstract. Cross-talk between G-protein-coupled receptor (GPCR) and epidermal growth factor receptor (EGFR) signaling systems is established in a wide variety of normal and neoplastic cell types. Here, we show that proteinase-activated receptor 1 (PAR_1) mediates the tyrosine phosphorylation of EGFR in human renal carcinoma cells expressing PAR_1 and PAR_3 endogeneously. This GPCR-EGFR signal transduction pathway cross-talk requires matrix metalloproteinase activity and is involved in the regulation of renal carcinoma cell migration across a collagen barrier as shown using a Boyden chamber type assay. Our data therefore document a regulatory role of PAR_1-mediated EGFR transactivation in cancer cell chemotactic migration. Further, our results underline the importance of PAR_1-mediated pathways in kidney cancer cells and suggest that the thrombin/PAR_1 system mediating EGFR transactivation may play a role in the progression of this tumor entity.

Introduction

The cellular effects of the serine proteinase thrombin (EC 3.4.21.5) are mediated at least in part by members of a novel subfamily of G-protein-coupled receptors designated proteinase-activated receptors (PARs) (reviewed in refs. 1-4). This receptor family comprises PAR_1 (5,6), PAR_3 (7) and PAR_4 (8), which are activated by thrombin, and PAR_2 (9), which may be activated by trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, and membrane-tethered serine proteinase-1.

Thrombin is generated from its precursor enzyme prothrombin in blood and has a critical function in blood coagulation. Therefore, initially much of the available information about thrombin receptors relates to vascular biology, and their function in thrombosis, inflammation, vascular injury and remodelling has been investigated intensively (reviewed in refs. 1-4). Wojtukiewicz *et al* demonstrated the expression of PAR_1 in carcinosarcoma and melanoma cells (10) suggesting a role of thrombin receptors in carcinogenesis. During the following years, thrombin receptors were characterized on signaling and cellular level in cells from different tumor entities including larynx (11), pancreas (12), brain (13), prostate (14), breast (15) and colon (16). The very extensive work done in this field over the past 5-10 years demonstrates for cancer that thrombin can signal via a diverse set of mechanisms and there is no single signal transduction pathway activated by thrombin in all situations. Rather, the data indicate that, in different tumor environments, thrombin can signal via distinct mechanisms at the cellular level. This diversity of signaling by thrombin is in keeping with the basic knowledge that every tumor seems to develop its own unique biology and underscores the necessity to evaluate the role of thrombin receptors in each tumor type.

In this context, it is important to note that renal cell carcinomas (RCCs) belong to the 'coagulation type' tumors wherein the tumor cells are associated with an intact coagulation pathway that leads to thrombin formation (17). Therefore, a role of thrombin and its proteolytically activated receptors (PARs) in RCCs under *in vivo* conditions is very likely. Given this potential role of PARs, we considered it of great importance to study PAR activation by thrombin and other PAR-activating ligands in renal cell carcinoma cells.

Recently we have demonstrated the expression of PAR_1- and PAR_3-type thrombin receptors in renal carcinoma cells (18,19). However, their impact on signaling and their influence

Correspondence to: Dr Roland Kaufmann, Research Laboratory, Department of General and Visceral Surgery, Research Center Lobeda, Medical Faculty at the Friedrich Schiller University Jena, Erlanger Allee 101, D-07747 Jena, Germany
E-mail: roland.kaufmann@med.uni-jena.de

Key words: proteinase-activated receptors, thrombin receptor, epidermal growth factor receptor, receptor transactivation, renal cell carcinoma

on the way these cells behave are yet to be evaluated in any depth. In the work we report here, we used the human renal carcinoma cell line, A-498, as well as primary renal cell cultures established from cases of surgically resected RCCs as cellular models. We tested the hypothesis that thrombin-induced activation of PAR_1 would transactivate the receptor for epidermal growth factor receptor (EGFR). The EGFR receptor tyrosine kinase is well known to be involved in the metastatic behaviour of kidney cancer cells (20). Given the potential impact of EGFR transactivation on metastasis, we also evaluated the potential role of thrombin/PAR_1-triggered EGFR transactivation in renal carcinoma A-498 cell migration.

Materials and methods

Reagents. Human α-thrombin (3085 NIH-U/mg protein) was purchased from Haemochrom Diagnostica Supplies (Essen, Germany) and the EGFR inhibitor, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (tyrphostin AG 1478), and the matrix metalloproteinase (MMP) inhibitor, N-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methyl-amide (GM-6001; galardin), were obtained from Calbiochem/Merck Biosciences (Bad Soden, Germany). The PAR_1-selective antagonist, SCH 79797, was purchased from Tocris Bioscience (Ellisville, MO, USA). The antibodies used were rabbit polyclonal anti-EGFR Ab-17 from Lab Vision/NeoMarkers (Fremont, CA, USA) and mouse monoclonal anti-phosphotyrosine antibody 4G10 from Upstate (Lake Placid, NY, USA). Secondary HRP-conjugated antibodies were goat anti-rabbit antibody (Santa Cruz, CA, USA) and goat anti-mouse antibody (Novagen/Merck Biosciences, Bad Soden, Germany).

Peptide synthesis. The peptide, TFLLRN, was synthesized by Fmoc strategy on an ABI-Peptide-Synthesizer 433A using TentaGel S RAM resin (capacity 0.26 mmol/g Rapp Polymere Tübingen). The side chains of the amino acids were protected by Trityl for Asn, t-Butyl for Thr and Pbf for Arg. The cleavage of the peptides from resin was performed with trifluoro acetic acid, 5% H_2O and 3% triisopropylsilane. The peptides were precipitated by ether and lyophilized. Purification of the crude peptide was carried out by using preparative HPLC on a 40x 300 mm Vydac C18-column with a flow rate of 100 ml per min under standard conditions. The correct mass was determined by MALDI mass spectrometry on a Voyager-DEPRO workstation.

Cell culture

A-498 cells. Human A-498 renal carcinoma cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, ACC 325) were routinely cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum at 37˚C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days. For subculturing, treatment with trypsin/EDTA was used. Since trypsin is a critical point in studies on PAR-type receptors, the cells have been re-fed sufficiently to remove all traces of trypsin.

Primary RCC cultures. Primary cultures were established from surgically resected specimens of primary renal cell carcinomas obtained at the Department of Urology as described (18). To confirm their epithelial nature and exclude myofibroblast contamination, cell cultures were tested by standard immunochemistry using a monoclonal anti-cytokeratin antibody (Dako, clone MNF116) and an anti-smooth muscle actin antibody (Dako, clone 1A4). Cells were cultured in Amniomax-100 (Invitrogen Corp.) at 37˚C, 5% CO_2 in a humidified incubator.

Cell lysis, immunoprecipitation and immunoblotting. Prior to lysis, cells were grown in 75-cm^2 tissue culture flasks (Greiner) at 80% confluency and serum-starved for 17 h. After treatment with the test agents, cells were kept on ice, scraped and collected by centrifugation, washed twice with PBS containing bacitracin (100 μg/ml), PMSF (0.1 mM), pepstatin A (1.0 μg/ml), leupeptin (2.0 μg/ml), Na-orthovanadate (1 mM) and NaF (1 mM), pH 7.4, and centrifuged again. The pellets were treated with lysis buffer [PBS, containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate and 0.1% (w/v) SDS] for 10 min on ice, resuspended and centrifuged at 13000 rpm for 20 min (4˚C). After protein quantification and calibration, supernatants were diluted with lysis buffer to an equal volume and immunoprecipitated overnight at 4˚C using a rabbit polyclonal anti-EGFR antibody and 30 μl protein A-sepharose. Precipitates were centrifuged and washed three times with lysis buffer, suspended in SDS sample buffer and subjected to gel electrophoresis on 7.5% gels. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. EGFR phosphorylation was detected by Western blot analysis with anti-phosphotyrosine monoclonal antibody 4G10. Secondary antibody was detected by using an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Corp.) by exposure to Kodak X-Omat films. In all of the experiments, the immunoblots were reprobed with an antibody to total EGFR protein to confirm equal protein loading. Before reprobing, nitrocellulose filters were stripped with a solution containing 100 mM mercaptoethanol and 2% SDS at 50˚C for 30 min.

Immunoreactive bands at 170 kDa for EGFR-specific tyrosine kinase were quantified by scanning densitometry (AIDA image analyzer version 3.22 program) and the area under the curve (AUC) was expressed as arbitrary intensity units.

Migration assay. Cell migration was tested using a 48-well boyden chamber (NeuroProbe, Inc., Gaithersburg, MD, USA). In the standard assay, 51 μl of A-498 cell suspension in DMEM without FCS ($4x10^5$ cells) with or without inhibitors were placed in each upper chamber well and 27 μl of DMEM without FCS containing the chemoattractant or vehicle in each lower well. Then, incubation for 24 h at 37˚C in a humidified incubator with 5% CO_2 was performed to allow cell migration through a polycarbonate filter (6.5 mm in diameter, 8-μm pore size) precoated with collagen. After the incubation period, the filter was removed, and its upper side was wiped gently with a cotton tip swab to remove non-migrated cells. The migrated cells on the lower surface of the membrane were fixed with 96% ethanol, stained with Giemsa solution, and counted under a Zeiss Axiolab microscope. Data were acquired from three independent experiments, wherein each performed in octuplicates.

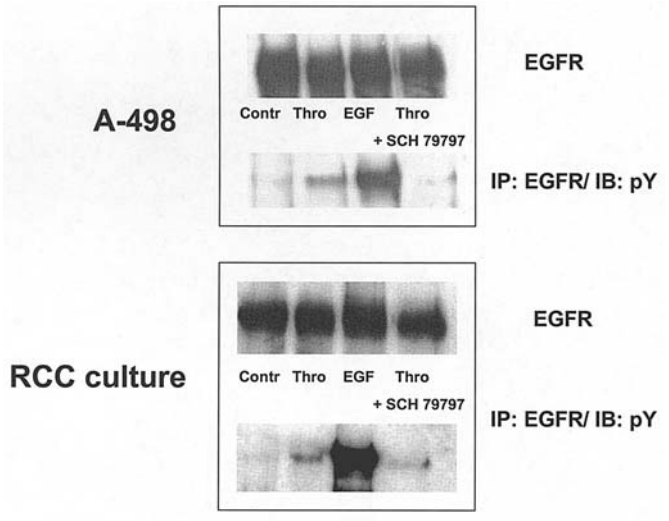

Figure 1. Thrombin and EGF induce the tyrosine phosphorylation of EGFR in A-498 cells and in cells from primary cultures established from surgically resected cases of human renal carcinomas and the PAR_1 antagonist, SCH 79797, blocks the effect of thrombin. Serum-starved cells were pretreated for 10 min with vehicle or SCH 79797 and then challenged with vehicle, thrombin (1.0 NHI-U/ml) or EGF (100 ng/ml) for 10 min at 37˚C. The extracts were subjected to immunoprecipitation (IP) with anti-EGFR, followed by immunoblot (IB) analysis with anti-phosphotyrosine; the blots were stripped and then reprobed for EGFR protein. A representative experiment is depicted. Results were reproduced by three independent experiments.

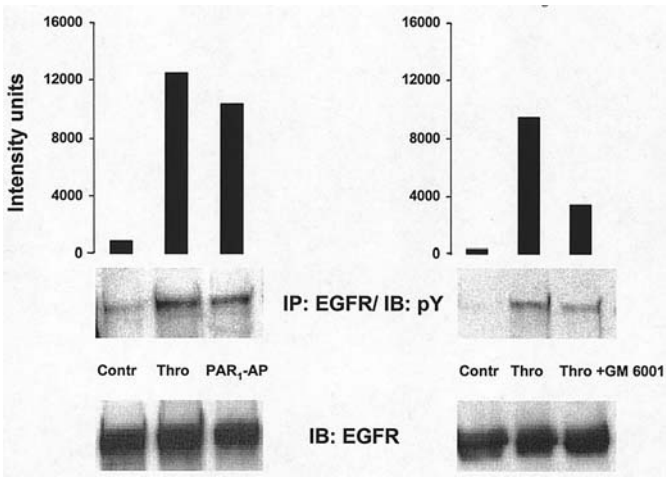

Figure 2. PAR_1 agonist TFLLRN mimicks and MMP inhibitor GM 6001 blocks the thrombin-induced tyrosine phosphorylation of EGFR in A-498 cells. Starved A-498 cells were pretreated for 10 min with vehicle and MMP inhibitor (10 μM), respectively, and then stimulated with thrombin (1.0 NHI-U/ml) for 10 min. EGFR tyrosine phosphorylation was determined by immunoprecipitation (IP) with anti-EGFR, followed by immunoblot (IB) analysis with anti-phosphotyrosine. The blots were stripped and then reprobed for EGFR protein. A representative experiment is shown. Equivalent results were obtained in three separate experiments. Data are expressed as intensity units obtained by densitometric analysis.

Protein assay. Protein was determined with BSA as standard as described elsewhere (21).

Statistical analysis. All results from migration experiments are expressed as mean ± SD for one experiment performed in octuplicate. Differences between data were tested by using the SPSS 13 for Windows computer program (SPSS Inc., Chicago, IL, USA). As the data were not normally distributed, non-parametric Mann-Whitney U-test was used. A p-value <0.05 was considered to be significant.

Results

PAR_1 mediates the tyrosine phosphorylation of EGFR. Since EGFR transactivation by GPCRs is a fundamental mechanism involved in the regulation of key cellular activities we evaluated its participation in PAR-type thrombin receptor signaling and action at the cellular level in human renal carcinoma cells.

As shown in Fig. 1, thrombin induced an enhanced tyrosine phosphorylation of the EGFR in cells of the permanent renal carcinoma cell line, A-498, and in primary cell cultures from surgically resected RCC specimens. This effect can be blocked by the selective PAR_1 antagonist, SCH 79797 (22) (Fig. 1). As a positive control, the cells were stimulated with epidermal growth factor (EGF), which is known to induce strong EGFR phosphorylation. As expected, EGF was able to increase the tyrosine phosphorylation of EGFR in both A-498 cells and primary RCC cultures (Fig. 1).

In further experiments, we used the receptor-selective synthetic PAR_1-activating peptide, TFLLRN, as a PAR_1 agonist. This peptide is well recognized for its ability to activate the PAR_1-type thrombin receptor without affecting

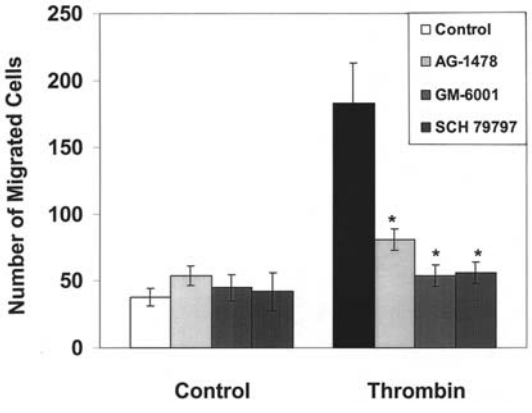

Figure 3. Thrombin promotes A-498 cell migration, which is inhibited by PAR_1 antagonist SCH 79797, MMP inhibitor galardin and tyrphostin AG-1478. Migration was tested in a modified Boyden-Chamber assay. The serum-starved cells were preincubated for 10 min with vehicle, SCH 79797 (1.0 μM), MMP inhibitor GM 6001 (10 μM) and tyrphostin AG-1478 (1.0 μM), respectively, and then placed in the upper part of the chamber. Cells were permitted to migrate to a polycarbonate membrane in response to thrombin (1.0 NHI-U/ml) added to the lower compartment of chamber. After 24 h, migrated cells were fixed, stained and quantified by microscopic counting. Bars represent the mean values ± SD of octuplicates obtained in one experiment, which is representative of three independent assays. *p-value = 0.002.

PAR 2 or 4. As shown in Fig. 2, TFLLRN mimicked the effect of thrombin in increasing the tyrosine phosphorylation of EGFR. This effect was also observed at lower TFLLRN concentrations (10 μM, 20 μM; data not shown).

Matrix metalloproteinase (MMP) activity is involved in the thrombin-induced tyrosine phosphorylation of EGFR. To examine whether matrix metalloproteinase activity might be involved in the transactivation of EGFR, we analysed the effect

of the matrix metalloproteinase inhibitor, galardin (GM-6001), a potent broad-spectrum hydroxamic acid inhibitor of MMPs (23) on the thrombin-induced tyrosine phosphorylation of EGFR in A-498 cells. For our experiments, we used the inhibitor at a concentration of 10 μM, which is known to inhibit completely almost all types of collagenases and gelatinases such as MMP-1, 2, 3, 8, and 9 (24-26). As shown in Fig. 2, 10 μM GM-6001 partly inhibited the effect of thrombin on the tyrosine phosphorylation of EGFR in A-498 cells. Even if not complete, this degree of inhibition was sufficient to block the transmigration of cells (Fig. 3).

The PAR_1-mediated effect on the migration of A-498 cells depends on EGFR. Using a boyden chamber with a filter precoated with collagen, we found significantly enhanced trans-well migration of A-498 cells stimulated with either thrombin (Fig. 3) or TFLLRN (data not shown) when compared with non-stimulated A-498 cells. Enhanced migration across a collagen-coated barrier was abolished by treatment with the PAR_1 selective antagonist, SCH 79797, tyrphostin AG-1478 or the MMP inhibitor, GM-6001 (Fig. 3).

Discussion

There is now considerable evidence that proteinase-activated receptor 1 (5,6), the prototype of a newly discovered G-protein receptor subfamily (3,27), plays an important role in the development and progression of different tumor entities including breast, colon, pancreas, lung, prostate and melanoma (28-30).

Here, we demonstrate signaling cross-talk between the PAR_1 thrombin receptor and EGFR in both cell-line and tumor-derived cultured renal carcinoma cells endogeneously expressing PAR_1 (18,19). Our results show that stimulation of cells from both the permanent cell line, A-498, and from primary RCC cultures with thrombin led to rapid tyrosine phosphorylation of EGFR. This effect was mimicked by the PAR_1-selective peptide, TFLLRN, and was inhibited by the PAR_1-selective antagonist, SCH 79797. Therefore, our results demonstrate that PAR_1 activation accounts for the thrombin-induced tyrosine phosphorylation of EGFR in renal carcinoma cells. Neither PAR_2 nor PAR_4 can be attributed to the effects of thrombin and the PAR_1-activating peptide; a) because thrombin is not capable of activating PAR_2 at concentrations of 1 NHI-U/ml, b) because the PAR_1 antagonist would not have blocked the activation of either PAR 2 or 4 at the concentrations used and c) because TFLLRN, at the concentrations used, cannot activate PAR_4 or PAR_1. In addition, PAR_3 would not appear to be a candidate for cross-activating EGFR since, in isolation, PAR_3 has yet to be shown to generate intracellular signals, either from thrombin or from an activating peptide such as TFLLR-amide (7).

With the PAR_1-driven transactivation of EGFR in renal carcinoma cells, we provide a further example of cross-talk between G-protein-coupled recepors (GPCRs) and EGFR that represents an important mechanism for combined GPCR-growth factor receptor signal transduction. This GPCR-mediated transactivation of EGFR may well play a key role in the regulation of essential normal cellular processes and in the pathophysiology of hyperproliferative diseases such as cancer (31).

In renal cell carcinoma, multiple studies have documented that the overexpression of EGFR and its ligands epidermal growth factor and transforming growth factor occurs frequently (32-34). EGFR-associated autocrine and paracrine loops are associated with the development and progression of RCC metastases (35,36). Therefore, EGFR signaling is considered as a key player in renal carcinogenesis. This signaling mechanism adds to other associated oncogenic events related to cytokine production, cell cycle control, anti-apoptotic signal machinery and angiogenesis (37). Due to its ability to transactivate EGFR, a significant regulatory role of PAR_1 in this scenario may be suggested. EGFR activation induced by GPCR agonists is believed to be caused by the metalloproteinase-mediated release of heparin-binding EGF, which in turn activates EGFR. This EGFR transactivation process can be blocked by a variety of broad-spectrum metalloproteinase inhibitors (38). In renal carcinoma cells, the matrix metalloproteinase inhibitor, GM 6001, diminished the tyrosine phosphorylation of the EGFR induced by thrombin. Thus, matrix metalloproteinase activity is critically involved in the PAR_1-mediated transactivation of EGFR in renal carcinoma cells.

While a similar mechanism, with the participation of MMPs, has been shown recently in HT-29 colon carcinoma cells where PAR_1 mediated enhanced cell proliferation by MMP-dependent EGFR transactivation (39), in a separate cell system (cardiac fibroblasts), PAR_1 activation results in EGFR transphosphorylation in an MMP-independent Src family kinase-dependent process (40). These distinct results imply that PAR_1 signaling is contextual in nature, depending on the cell type in which the EGFR and PAR_1 reside. Since colon and kidney carcinoma are tumors from epithelial origin, it is important to elucidate whether MMP-dependent PAR_1-EGFR cross-talk represents a common pathway in epithelial tumors.

Activation of EGFR has been identified as an important feature that initiates the cascade of intracellular signaling events that regulate cell proliferation, survival, angiogenesis, cell movement, and metastasis (31). In A-498 renal carcinoma cells, we found that thrombin and the PAR_1-activating peptide, TFLLRN, significantly enhanced cell migration through a collagen-coated barrier. This effect was completely blocked by pretreatment of the cells with the PAR_1-selective antagonist, SCH 79797; the MMP inhibitor, galardin; or the selective EGFR kinase inhibitor, tyrphostin AG-1478. Collectively, these data demonstrate that PAR_1 accounts for thrombin's effect on A-498 cell migration and that MMP-dependent EGFR activation is critically involved. To our knowledge, our report is the first to define a role for PAR_1-mediated EGFR transactivation in the enhanced chemotactic trans-well migration of cancer cells.

In summary, our study describes an important receptor cross-talk mechanism in renal carcinoma cells involving thrombin-mediated transactivation of EGFR by the PAR_1-type thrombin receptor. This mechanism is dependent on matrix metalloproteinase activity and is involved in renal carcinoma cell migration. On the basis of our findings, one can suggest that inhibiting all three of these therapeutic targets, PAR_1,

MMP and EGFR, and thrombin itself simultaneously may provide a novel approach for renal cell carcinoma.

Acknowledgements

The invaluable technical assistance provided by Beate Schulze, Elke Oswald and Margit Saupe is grateful acknowledged. This study was supported by a grant from the Interdisciplinary Center for Clinical Research (ICCR) Jena.

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