SPANDIDOS PUBLICATIONS

Green tea polyphenol epigallocatechin-3-gallate inhibits thrombin-induced hepatocellular carcinoma cell invasion and p42/p44-MAPKinase activation

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Received December 30, 2008; Accepted February 4, 2009

DOI: 10.3892/or_00000349

Abstract. Thrombin has been recently demonstrated to promote hepatocellular carcinoma (HCC) cell migration by activation of the proteinase-activated receptor (PAR) subtypes PAR_1 and PAR_4 suggesting a role of these proteinase-receptor systems in HCC progression. In this study, we investigated the effect of (-)-epigallocatechin-3-gallate (EGCG), the major polyphenolic compound of green tea on thrombin-PAR_1/PAR_4-mediated hepatocellular carcinoma cell invasion and p42/p44 MAPKinase activation. In this study we used the permanent liver carcinoma cell line HEP-3B and two primary cultures established from surgically resected HCCs. We found that stimulation of HCC cells with thrombin, the PAR_1-selective activating peptide, TFLLRN-NH_2, and the PAR_4-selective activating peptide, AYPGKF-NH_2, increased cell invasion across a Matrigel-coated membrane barrier and stimulated activation of p42/p44 MAPKinase phosphorylation. Both the effects on p42/p44 MAPKinases, and on cell invasiveness induced by thrombin and the $PAR_{1/4}$ subtype-selective agonist peptides were effectively blocked by EGCG. The results clearly identify EGCG as a potent inhibitor of the thrombin-PAR_1/PAR_4-p42/p44 MAPKinase invasive signaling axis in hepatocellular carcinoma cells as a previously unrecognized mode of action for EGCG in cancer cells. Moreover, the results suggest that (-)-epigal-locatechin-3-gallate might have therapeutic potential for hepatocellular carcinoma.

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Key words: thrombin, proteinase-activated receptors, PARs, thrombin receptor, epigallocatechin-3-gallate, hepatocellular carcinoma

Introduction

Several individual constituents of thrombin-generating pathways have been implicated in neoplasia including coagulation factors VIIa, IXa, Xa and thrombin (IIa) (1-5). Thrombin in particular has multiple cellular effects including induction of cell proliferation and motility, enhancement of vascular permeability, deposition of matrix fibrin, promotion of tumor cell seeding, adhesion to endothelium and extracellular matrix and enhancement of the metastatic capacity of tumors (6,7). These cellular effects of thrombin are mediated at least in part by proteinase-activated receptors (PARs), a subfamily of G protein-coupled receptors (reviewed in refs. 8-10). Among the PAR family members, PAR_1 (11,12), PAR_3 (13) and PAR_4 (14) are targeted mainly by thrombin. PAR_2 (15) can be activated by trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, human kallikrein-related peptidases and membrane-tethered serine proteinase-1/matriptase 1, but not by thrombin (9,10). In the setting of cancer, the ability of thrombin to act via PARs was highlighted by the demonstration of PAR_1 expression in carcinosarcoma and melanoma cells (16), and during the last few years, growing evidence for a function of the PAR family in neoplasia has been obtained (17). Especially for PAR_1, a role in the progression of epithelial tumors including breast (18-20), colon (21) and kidney (22) has been shown.

Hepatocellular carcinoma (HCC) is a frequent malignancy worldwide (23) with an extremely poor prognosis, mainly because of HCC recurrence and metastases (24-26). Development and spreading of this tumor entity that are known to be accompanied with complex variations on molecular and cellular level have been studied extensively (reviewed in ref. 27). The understanding of the pathogenesis of HCC is still incomplete, and further studies in this field are warranted to find novel HCC treatment principles.

We have recently found that PAR_1 and PAR_4 coordinately regulate thrombin-induced hepatocellular carcinoma cell migration with the involvement of different intracellular effector systems including p42/p44 MAPkinases. Therefore, a role for the thrombin-$PAR_{1/4}$-triggered signaling in HCC progression has been suggested (28). In this context, it is

important to note that hepatocellular carcinoma (HCC) belongs to the 'coagulation type' tumors, in which there is an important role for thrombin formation within the tumor microenvironment (29,30). Therefore, a role of thrombin and its proteolytically activated receptors in HCC under *in vivo* conditions is very likely.

In the current study we assessed the role of (-)-epigallocatechin-3-gallate (EGCG), an active and major constituent of green tea. This compound has been shown earlier to possess anti-carcinogenic properties in various tumor entities including HCC (31-33). To assess the effect of EGCG on thrombin-induced invasive signaling in hepatocellular carcinoma cells, we used the human permanent HCC cell line HEP-3B, and primary cultures established from surgically resected specimen of primary HCCs. We performed experiments on cell invasion across a Matrigel transmembrane barrier and on activation of p42/p44 MAPKinase phosphorylation. To estimate the involvement of the proteinase-activated receptors PAR_1 and PAR_4, we elucidated whether EGCG was able to inhibit the invasive effect of PAR subtype-selective peptide agonists (PAR-APs). The results revealed EGCG as a novel antagonist of PAR signaling.

Materials and methods

Reagents. Human α-thrombin (3085 NIH-U/mg protein) was purchased from Haemochrom Diagnostica Supplies (Essen, Germany), epigallocatechin-3-gallate [(-)-cis-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol 3-gallate] was from Sigma-Aldrich Chemie GmbH (Munich, Germany). The PAR_1-selective antagonist SCH 79797 [(N-3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7H-pyrrolo[3,2-f]quinazoline-1,3-diamine)] was purchased from Tocris Bioscience (Ellisville, MO, USA), the PAR_4-selective antagonist trans-cinnamoyl-YPGKF-amide from Peptides International (Lousville, KY, USA). Mouse monoclonal phosphospecific antibody to p42/p44 MAPKinase and polyclonal anti-p42/p44 MAPKinase antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Permanent liver carcinoma cell line HEP-3B. Human HEP-3B liver carcinoma cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, ACC 325 and ACC 141) were routinely cultured in RPMI-1640 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 itself activates PAR-type receptors the subcultured cells were re-fed sufficiently to remove all traces of trypsin.

Primary hepatocellular carcinoma (HCC) cultures. Primary cultures, PHC1 and PHC2, were established from surgically resected specimens of primary hepatocellular carcinomas from patients who underwent surgery in the Department of General, Visceral and Vascular Surgery as described (34). To confirm their epithelial nature and to exclude myofibroblast contam-ination, cell cultures were analyzed by a standard immunochemistry approach, using a monoclonal anti-cytokeratin antibody (DacoCytomation GmbH, Germany, clone MNF116) and an anti-smooth muscle actin antibody (DacoCytomation GmbH, clone 1A4). Cells were cultured in Amniomax-100 (Invitrogen Corporation) at 37˚C and 5% CO_2 in a humidified incubator.

Peptide synthesis. The peptides TFLLRN-NH_2 and AYPGKF-NH_2 were synthesized by Fmoc strategy on an ABI-Peptide-Synthesizer 433A using TentaGel S RAM resin (capacity 0.24 mmol/g Rapp Polymere, Tübingen). The cleavage of the peptides from resin was performed with trifluoro acetic acid, 5% H_2O und 3% triisopropylsilane. The peptides were precipitated by ether and lyophilized. Purification of the crude peptide was carried out by using preparative HPLC on a 50x250 mm Kromasil C18-column with a flow rate of 100 ml per minute under standard conditions. The correct mass was determined by MALDI mass spectrometry on a Voyager-DEPRO workstation.

Preparation of cell lysates. The cells were collected by centrifugation at 1,000 x g for 5 min (4˚C), washed with PBS containing bacitracin (100 μg/ml), PMSF (0.1 mM), pepstatin A (1.0 μg/ml) and leupeptin (2.0 μg/ml), pH 7.4, and centrifuged again. The pellet was treated with lysis buffer (PBS, containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate and 0.1% (w/v) SDS for 30 min at 4˚C, resuspended and centrifuged at 30,000 x g for 15 min (4˚C).

Western blotting. Proteins of cell lysates were separated on a 12% SDS/PAGE and transferred to nitrocellulose membranes (BioRad). After blocking in 1% BSA/1% skimmed milk for 1 h, the nitrocellulose strips were incubated overnight with the first antibody. Strips were washed 2 times with 0.05% (v/v) Tween-20 washing buffer, incubated for 45 min with the secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and washed again 2 times as described above. In all of the experiments, the immunoblots were stripped and reprobed with antibodies to total protein to confirm equal protein loading. Secondary antibodies were detected by using chemiluminescence (ECL) Western blotting detection system (Amersham) by exposure to Kodak X-Omat film.

Protein assay. Protein was determined using the DC protein assay system from BioRad Laboratories according to the manufacturer's instructions.

Invasion assay. Tumor cell invasion was measured using a 48-well boyden chamber (NeuroProbe, Inc., Gaithersburg MD, USA). This assay was employed since it has been demonstrated that invasiveness in the assay correlates with the metastatic potential of a given cell line *in vivo* (36). HCC cell suspension (51 μl) ($4x10^5$ cells in the respective serum-free medium) with or without EGCG were placed in each upper chamber well and 27 μl of cell culture medium containing thrombin, the respective peptide agonist or vehicle in each lower well. Then, incubation for 48 h at 37˚C in a humidified incubator with 5% CO_2 was performed to allow cell invasion through a polycarbonate filter (6.5 mm in diameter, 8-μm pore size) precoated with solubilized tissue

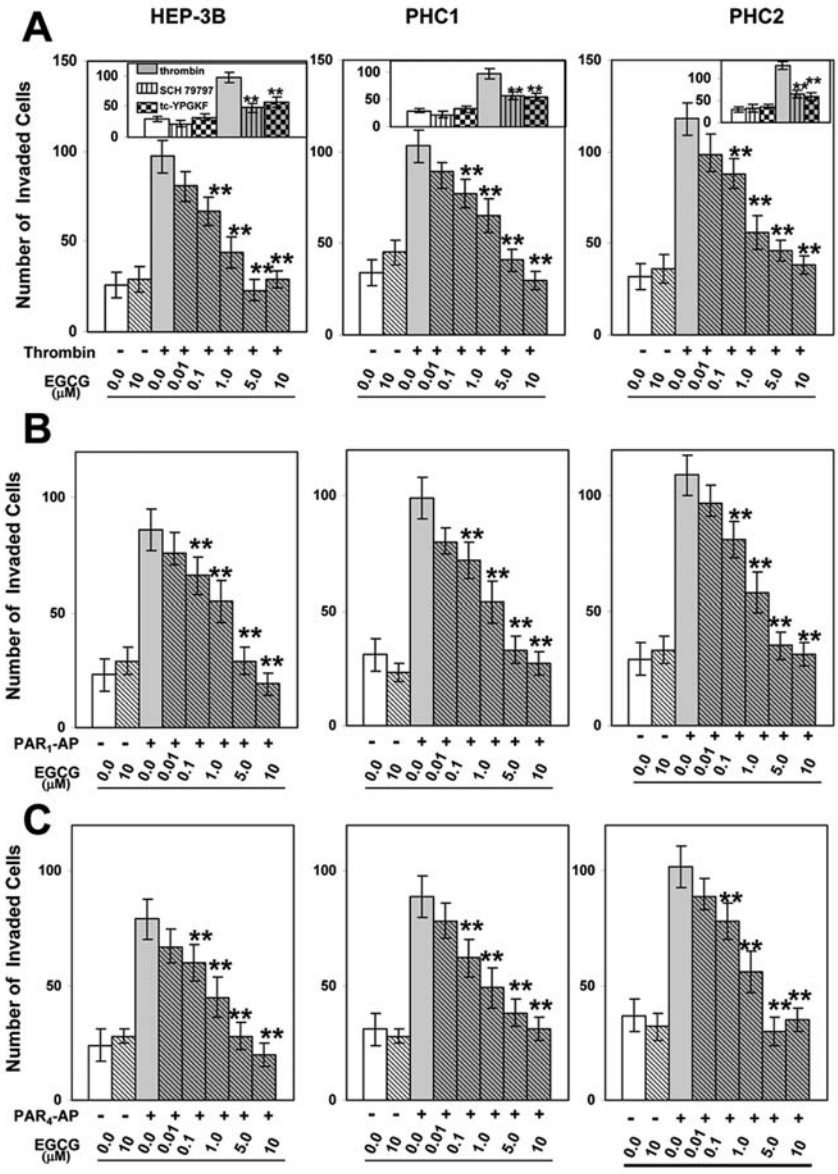

Figure 1. EGCG inhibits the effect of thrombin, the PAR_1-selective agonist peptide, TFLLRN-NH_2, and the PAR_4-selective agonist, AYPGKF-NH_2, on invasion of human hepatocellular carcinoma cells. HCC cell invasion was evaluated in a modified Boyden-Chamber assay. Cells (without serum and supplement, respectively) were permitted to invade through a Matrigel-coated polycarbonate membrane in response to (A) thrombin (1.0 NHI-U/ml), (B) TFLLRN-NH_2 (100 μM) or (C) AYPGKF-NH_2 (400 μM), added to the lower compartment of the chamber. Before stimulation cells were preincubated for 30 min with vehicle and EGCG, respectively. After 48 h, cells that invaded across the Matrigel were fixed, stained and quantified by microscopic counting. Bars represent the mean values + SD of octuplicates obtained in one experiment, which is representative for three independent assays. **P<0.05 vs. agonist stimulated. Inset (1A-C) the PAR_1-selective antagonist SCH 79797 and the PAR_4-selective antagonist trans-cinnamoyl-YPGKF-NH_2 inhibit the effect of thrombin on cell invasion. The cells serum-starved for 17 h were preincubated for 10 min with vehicle, SCH 79797 (10 μM) and trans-cinnamoyl-YPGKF-NH_2 (400 μM), respectively, and then the invasion assay was performed as described. (SCH 79797=PAR_1-selective antagonist SCH 79797; tc-YPGKF=PAR_4-selective antagonist, trans-cinnamoyl-YPGKF-NH_2). Bars represent the mean values ± SD of octuplicates obtained in one experiment, which is representative for three independent assays. **P<0.05 vs. thrombin-stimulated.

basement membrane (Matrigel; BD Biosciences Discovery Labware, Bedford, MA, USA). 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, involving octuplicate measurements for each condition. To exclude effects by cell proliferation control experiments with the proliferation inhibitor, mitomycin (10 μg/ml, 3 h preincubation), were performed.

Estimation of cell viability. For testing the effect of EGCG on HCC cell viability cells were seeded into 24-well plates and treated with the respective inhibitor for 48 h. Then the viable cells were counted by trypan blue dye exclusion test.

Statistical analysis. All results from migration experiments are expressed as means ± SD for one experiment performed

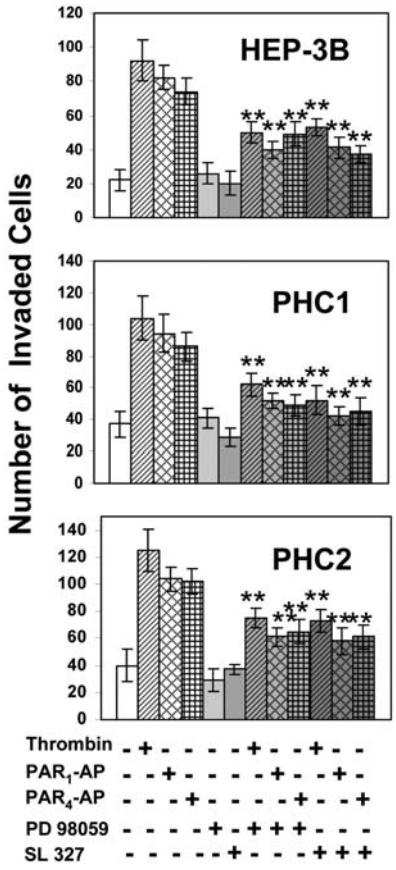

Figure 2. Inhibition of thrombin-PAR_1/PAR_4-stimulated invasion of HCC cells is mediated by p42/p44 MAPKinase activation. Serum-starved HEP-3B cells, cells from PHC-1 and PHC-2 cells, respectively, were plated on top of a Matrigel-coated polycarbonate membrane. Cells were preincubated for 30 min with vehicle, MEK inhibitor, SL 372 (5.0 μM), and MEK inhibitor, PD 98059 (10 μM), respectively. Cell invasion in response to the respective stimulus was analysed after 48 h, as described in the legend for Fig. 1. Representative results from three independent experiments are shown. $^{**}P<0.05$ vs. non stimulated control.

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. $P<0.05$ was considered to be significant.

Results

EGCG inhibits thrombin-induced increase in HCC cell invasion mediated by PAR_1 and PAR_4. A modified Boyden chamber Matrigel invasion assay with cells from the permanent HCC cell line HEP-3B and primary HCC cultures, established from surgically resected hepatocellular carcinomas was performed to determine if EGCG was capable of inhibiting thrombin-PAR-induced invasion. As demonstrated in Fig. 1A, thrombin (1.0 NHI-U/ml) significantly enhanced HCC cell invasion across the Matrigel barrier. To elucidate the PAR-subtype(s) involved in the thrombin-triggered stimulation of HCC cell invasion, we performed experiments with PAR-subtype selective agonists and antagonists (SCH 79797 for PAR_1 and trans-cinnamoyl-YPGKF-NH_2 for PAR_4). Both the PAR_1 selective agonist

Table I. EGCG (10 μM) inhibits the effect of thrombin, the PAR_1 -selective peptide TFLLRN-NH_2 and the PAR_4-selective peptide AYPGKF-NH_2 on p42/p44 MAPKInase activation in HCC primary cultures.

Cell culture	p42/p44 MAPKinase		
	Thro	PAR_1	PAR_4
PHC1	91.6±8.3	84.3±12.4	89.0±7.4
PHC2	88.5±10.8	85.5±11.9	93.0±11.4

Thro, % Inhibition ± SD of the effect of thrombin (1.0 NHI-U/ml); PAR_1, % Inhibition ± SD of the effect of TFLLRN-NH_2 (100 μM); PAR_4, % Inhibition ± SD of the effect of AYPGKF-NH_2 (400 μM); Data are from three independent experiments.

TFLLRN-NH_2 (100 μM; Fig. 1B) and the PAR_4 selective agonist AYPGKF-NH_2 (400 μM; Fig. 1C) significantly enhanced the invasive capacity of cells from the permanent HCC cell line HEP-3B and from primary HCC cultures, PHC1, PHC2, across Matrigel. The receptor-inactive control peptides, NRLLFT-NH_2 for PAR_1, and YAPGKF-NH_2 for PAR_4, were unable to induce an effect on hepatocellular carcinoma cell invasion (data not shown), thereby unspecific effects of the peptide sequences may be excluded. For subtype selective receptor inhibition, the PAR_1-selective antagonist SCH 79797 [10 μM; (36)] and the PAR_4-selective antagonist, trans-cinnamoyl-YPGKF-NH_2 [400 μM; tcY-NH_2; (37)], were used. As demonstrated in the insets of Fig. 1A-C, both SCH 79797 and trans-cinnamoyl-YPGKF-NH_2 reduced thrombin's stimulatory action on the HCC cell invasive capacity.

Pretreatment of the cells for 30 min with EGCG inhibited the effect of thrombin (Fig. 1A) and the PAR-selective agonist peptides (Fig. 1B and C) on HCC cell invasion in a concentration-dependent manner. It is important to note that EGCG concentrations as low as 1.0 μM, close to physiologically relevant level observed after dietary or supplemental intake (38-40) had significant inhibition capacity. In addition, 0.01-10 μM concentrations of EGCG did not inhibit basal levels of HCC cell invasion compared with untreated control (Fig. 1A-C) and had no significant effect on cell viability as evaluated by trypan blue exclusion test (data not shown).

EGCG inhibits the effect of thrombin, TFLLRN-NH_2 and AYPGKF-NH_2 on p42/p44 MAPKinase activation that is involved in thrombin/$PAR_{1/4}$-stimulated HCC cell invasion. To explore the effect of EGCG on intracellular signaling level in HCC cells, we focused on p42/p44 MAPKinases that are known to be a critically involved in hepatocarcinogenesis (reviewed in refs. 41 and 42). Using the MEK inhibitors, PD 98059 and SL 327, both inhibited the effect of either, thrombin or the PAR_1 selective agonist peptide, TFLLRN-NH_2, or the PAR_4 selective agonist AYPGKF-NH_2 on HEP-3B cell invasion (Fig. 2) indicating the involvement of p42/p44

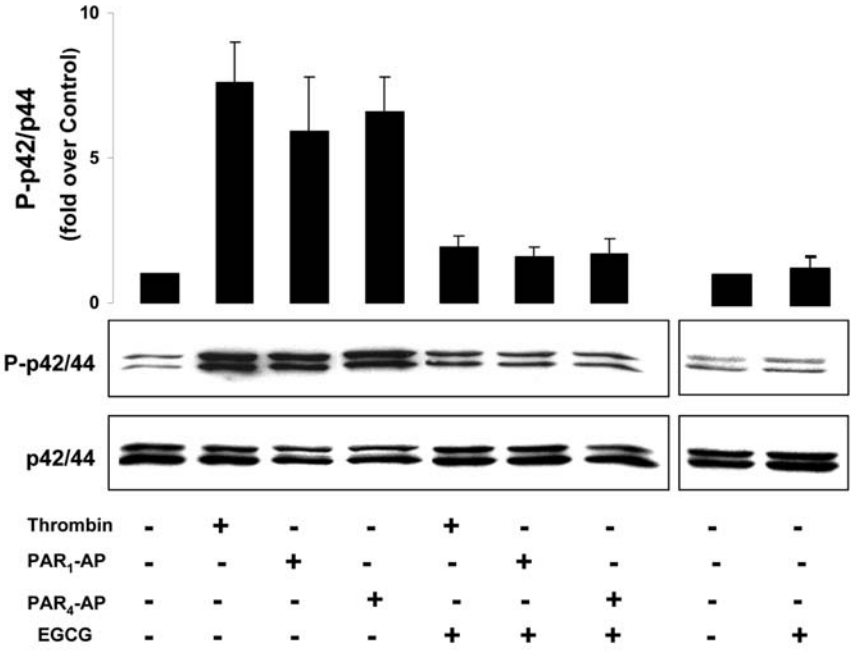

Figure 3. PAR_1-AP TFLLRN-NH_2 and PAR_4-AP AYPGKF-NH_2 induce activation of p42/44 MAPKinase in HEP-3B cells. Serum-starved HEP-3B cells were treated with thrombin (1.0 NIH-U/ml), PAR_1-AP TFLLRN-NH_2 (100 μM) or PAR_4-AP AYPGKF-NH_2 (400 μM) for 20 min. The cell lysates were subjected to SDS-PAGE and Western blotting with an anti-phospho-p42/44 MAPKinases antibody. Immunoblot analysis from a representative experiment is shown with total p42/44 MAPKinase as control for constant protein loading in all lanes. The data are expressed as the fold increase over untreated control (mean ± SD) from three independent experiments in the histograms above the blots. P-p42/44 = phosphorylated p42/44 MAPKinases.

MAPKinases in thrombin-PAR_1/PAR_4 invasive effect. Similar results were observed in cells from the HCC primary cultures, PHC1 and PHC2 (Fig. 2).

Stimulation of HEP-3B cells with the PAR_1 selective agonist peptide, TFLLRN-NH_2, or the PAR_4 selective agonist AYPGKF-NH_2, for 20 min caused a significant increase in phospho-p42/p44 MAPKinases, consistent with a concurrence of MAPKinase activation (Fig. 3). In keeping with its ability to activate PAR_1 and PAR_4, thrombin also induced a strong increase in phosphorylation of p42/p44 MAPKinases in HEP-3B cells (Fig. 3). As seen in Fig. 3, EGCG (10 μM) potently inhibited the increase in p42/p44 activation in HEP-3B cells induced by thrombin, the PAR_1 activating peptide TFLLRN-NH_2 and the PAR4 activating peptide AYPGKF-NH_2, respectively. As further demonstrated, EGCG (10 μM) by itself had no significant effect on MAPKinase phosphorylation basal level in HEP-3B liver carcinoma cells (Fig. 3). A comparable inhibitory effect of EGCG on p42/p44 activation could be observed in cells from the primary HCC cultures, PHC1 and PHC2 (Table I).

Discussion

In this study we found that (-)-epigallocatechin-3-gallate, an active and major constituent of green tea, inhibits the PAR_1/PAR_4-mediated effect of the serine proteinase thrombin on HCC cell invasiveness and p42/p44 MAPKinase activation. In cells from the permanent HCC cell line HEP-3B and cells from primary cultures established from surgically resected hepatocellular carcinoma specimens thrombin stimulates cell invasion by interaction with PAR subtypes, PAR_1 and PAR_4. Our conclusions are based on the ability of the PAR-targeted agonists for PARs 1 and 4 to mimic the actions of thrombin and the block of thrombin action by the two PAR antagonists, SCH79797 for PAR_1 (36), and trans-cinnamoyl-YPGKF-NH_2 for PAR_4 (37). The receptor-inactive PAR-AP sequence-related peptides (NRLLFT-NH_2 for PAR_1; YAPGKF-NH_2 for PAR_4) did not affect HCC cell invasion, thereby establishing further the receptor selectivity of the PAR-activating peptides we used to activate PARs 1 and 4.

As the main finding of the study we report that EGCG is an inhibitor of the thrombin-stimulated cell invasion in HCC cells mediated by the proteinase activated receptors, PAR_1 and PAR_4. This was concluded since EGCG inhibited both the effect of the endogeneous PAR agonist thrombin and selective PAR subtype selective agonists (TFLLRN-amide for PAR_1 and AYPGKF-amide for PAR_4) that mimicked the effect of proteolytic PAR activation by thrombin. Moreover, EGCG inhibited the thrombin-PAR_1/PAR_4-induced phosphorylation-activation of p42/p44 MAPKinases, and inhibition of these kinases by pharmacological agents (PD 98059 and SL327) resulted in an inhibition of thrombin-PAR_1/PAR_4-stimulated invasive capacity of HCC cells. Therefore, it may be concluded that EGCG inhibits PAR_1/PAR_4-triggered invasion via inhibition of p42/p44 MAPKinases. Our conclusion is based on the experiments demonstrating that thrombin, the PAR_1-AP and the PAR_4-AP strongly increased the phosphorylation of p42/p44 MAPKinases and EGCG inhibited thrombin-PAR_1/PAR_4-stimulated p42/p44 MAPKinase phosphorylation in HCC cells. Therefore, it seems that p42/p44 MAPKinases activation may be required for the thrombin-PAR_1/PAR_4-stimulated HCC cell invasion.

This hypothesis may be further strengthened by the observation that PD98059 and SL327, selective inhibitors of p42/p44 MAPKinases, decreased the invasive effect of thrombin and the PAR agonist peptides.

(-)-Epigallocatechin-3-gallate is known to possess anti-oxidant (43), anti-proteolytic (44,45), and anti-proliferative activity (46). More detailed, *in vivo* mouse studies have established that EGCG can function as a strong chemo-preventative agent against cancer development and progression (47,48), and epidemiological and preclinical studies have demonstrated that catechins derived from green tea have profound chemopreventative and antitumor effects (38,48,49). The mechanisms by which EGCG is able to produce these effects is wide-ranging and includes e.g. inhibition of growth factor signaling pathways and numerous kinases JNK, AP-1, p44/p42 MAPK (for reviews see e.g. 38,50).

With our study on hepatocellular carcinoma cells we have identified a novel mechanism for the anti-carcinogenic effects of EGCG, the inhibition of thrombin induced cancer cell invasion. Since the ability of cancer cells to invade is one of the hallmarks of the metastatic phenotype and more specifically, post-extravasation cell migration and invasion is known to be critical for the metastatic process in hepato-cellular carcinoma (51), EGCG may have therapeutic potential for hepatocellular carcinoma.

Acknowledgements

We would like to thank Beate Schulze, Elke Oswald and Gabriele Weigand, Research Lab., Department of General, Visceral and Vascular Surgery, for the excellent technical assistance.

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