Activation of Proteinase-Activated Receptor 1 Promotes Human Colon Cancer Cell Proliferation Through Epidermal Growth Factor Receptor Transactivation

Dalila Darmoul,1 Valérie Gratio,1 Hélène Devaud,1 Franck Peiretti,2 and Marc Laburthe1

1Neuroendocrinologie et Biologie Cellulaire Digestives, Institut National de la Sante et de la Recherche Medicale, Paris, France and 2UMR 626, Faculté de Médecine Timone, Marseille, France

Abstract
Serine proteases are now considered as crucial contributors to the development of human colon cancer. We have shown recently that thrombin is a potent growth factor for colon cancer cells through activation of the aberrantly expressed proteinase-activated receptor 1 (PAR1). Here, we analyzed the signaling pathways downstream of PAR1 activation, which lead to colon cancer cell proliferation in HT-29 cells. Our data are consistent with the following cascade of events on activation of PAR1 by thrombin or specific activating peptide: (a) a matrix metalloproteinase–dependent release of transforming growth factor-α (TGF-α) as shown with TGF-α blocking antibodies and measurement of TGF-α in culture medium; (b) TGF-α–mediated activation of epidermal growth factor receptor (EGFR) and subsequent EGFR phosphorylation; and (c) activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and subsequent cell proliferation. The links between these events are shown by the fact that stimulation of cell proliferation and ERK1/2 on activation of PAR1 is reversed by the MMP inhibitor batimastat, stimulation of cell proliferation and ERK1/2 on activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and subsequent cell proliferation. The links between these events are shown by the fact that stimulation of cell proliferation and ERK1/2 on activation of PAR1 is reversed by the MMP inhibitor batimastat, TGF-α neutralizing antibodies, EGFR ligand binding domain blocking antibodies, and the EGFR tyrosine kinase inhibitors AG1478 and PD168393. Therefore, transactivation of EGFR seems to be a major mechanism whereby activation of PAR1 results in colon cancer cell growth. Finally, PAR1 activation induces Src phosphorylation, which is reversed by using the Src tyrosine kinase inhibitor PP2, suggesting that Src activation plays a permissive role for PAR1-mediated ERK1/2 activation and cell proliferation probably acting downstream of the EGFR. These data explain how thrombin exerts robust trophic action on colon cancer cells and underline the critical role of EGFR transactivation. (Mol Cancer Res 2004;2(9):514–22)

Introduction
Besides their contribution to cancer progression by the degradation of extracellular matrix proteins, it is now clear that some proteases serve as signal molecules controlling cell functions through specific membrane receptors, the protease-activated receptors (PAR; refs. 1–4). The recent discovery of a family of PARs, which are receptors for a variety of serine proteases, has changed the traditional view on the role of serine proteases in biology. PARs are seven-transmembrane spanning domain G protein-coupled receptors (GPCR) comprising four receptors named PAR1, PAR2, PAR3, and PAR4 (5, 6). Thrombin is the physiologic activator of PAR1, PAR3, and PAR4, whereas PAR2 is activated by multiple trypsin-like enzymes including trypsin and mast cell tryptase but not by thrombin. The mechanism of activation of PARs was initially established for PAR1 (7) and seems to be a paradigm for other PARs (5, 6, 8). They are irreversibly activated by a proteolytic mechanism in which the protease binds to and cleaves the NH2-terminal exodomain of the receptor. This cleavage generates a new NH2-terminal sequence that binds intramolecularly to the core receptor and serves as a tethered ligand. Synthetic activating peptides (AP) that mimic the tethered ligand domains of PAR1, PAR2, and PAR4 have been developed. The activation of PARs by these synthetic APs is independent of receptor proteolysis (5, 6, 8).

The expression of PAR1, the predominant thrombin receptor, has been correlated with the malignant phenotype in colon cancer. Indeed, this receptor is absent in normal human colon epithelial cells but aberrantly expressed during colonic carcinogenesis (4). Activation of the ectopic PAR1 in colon cancer cells induces proliferation and motility (4). These findings supported the idea that PAR1 and its physiologic activator thrombin should now be considered as crucial contributors to the development of human colon cancer. However, the cellular mechanisms underlying PAR1-induced colon cancer cell proliferation have not been elucidated yet.

By using the human colon cancer cell line HT-29 as a model (4), we showed here that activation of PAR1 by thrombin or synthetic AP results in a series of events that includes matrix metalloproteinase (MMP)–dependent release of transforming growth factor-α (TGF-α), transactivation of the epidermal growth factor (EGF) receptor (EGFR), and subsequent activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). These data represent a breakthrough in understanding of the role of PAR1 in controlling cell proliferation in colon cancer. They also highlight the central role of the EGFR as a pivotal downstream integrator in the control of colon cancer cell proliferation by serine proteases.

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Requests for reprints: Dalila Darmoul, Neuroendocrinologie et Biologie Cellulaire Digestives, Institut National de la Sante et de la Recherche Medicale U410, Faculté de Médecine Xavier Bichat, 75018 Paris, France. Phone: 33-1-44-85-61-30; Fax: 33-1-42-28-87-65. E-mail: darmoul@bichat.inserm.fr

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Results

Activation of ERK and Cell Proliferation Induced by PAR1 Agonists Are Attenuated by EGFR Tyrosine Kinase Inhibitors

We determined whether PAR1-mediated ERK activation and stimulation of colon cancer cell proliferation are dependent on EGFR kinase activity. Our previous studies have shown that half-maximal mitogenic response was obtained for thrombin (~3 nmol/L) and AP1 (50 μmol/L). The maximum of phosphorylation for the two agonists was reached within 5 to 10 minutes (4). HT-29 cells were stimulated for 5 minutes with AP1 (100 μmol/L) in the presence or absence of the EGFR kinase inhibitor tyrphostin AG1478 (3 μmol/L) and analyzed for ERK phosphorylation. As shown in Fig. 1A, the EGFR kinase inhibitor AG1478 abrogated the effects of PAR1 agonists on ERK phosphorylation in HT-29 cells. EGF was used as a positive control to show the specificity of the inhibitor. To determine whether the PAR1 agonist–induced EGFR activation is a general phenomenon for colon cancer cells, we treated two other colon cancer cells lines (T84 and Caco2) with PAR1 AP in the presence of the EGFR kinase inhibitor AG1478 and analyzed downstream signaling. AP1 treatment significantly increased ERK1/2 phosphorylation in both cell lines (Fig. 1B and C) and this AP1 effect was significantly attenuated by EGFR kinase inhibitor AG1478 (Fig. 1B and C). To ascertain the implication of EGFR kinase in mitogenic pathway induced by PAR1, we inhibited EGFR kinase activity with specific inhibitors and measured HT-29 cell proliferation. As shown in Fig. 2, AG1478 (3 μmol/L) significantly reduced PAR1-mediated cell proliferation in HT-29 cells. Because inhibition with AG1478 was only partial, we used another inhibitor of the EGFR tyrosine kinase, which was shown to be irreversible and more selective (i.e., PD168393; ref. 9). As shown in Fig. 2, this inhibitor completely abolished PAR1-mediated cell proliferation. These data support the involvement EGFR tyrosine kinase activity in ERK activation and subsequent proliferative responses induced by activation of PAR1.

Activation of PAR1 Promotes the EGFR Phosphorylation

Because PAR1-mediated activation of ERK and cell proliferation could be influenced by inhibitors of the EGFR tyrosine kinase, we sought to identify whether the activation of PAR1 results in EGFR tyrosine phosphorylation. HT-29 cells were treated with thrombin, AP1, or EGF for 5 minutes and the cells were lysed and assayed for tyrosine-specific phosphorylation of the EGFR. The EGFR in HT-29 cells became highly phosphorylated in response to EGF stimulation, whereas PAR1 agonist treatment resulted in moderate EGFR tyrosine phosphorylation (Fig. 3A). Further, we showed that inhibition of the EGFR tyrosine kinase activity by AG1478 abolished the PAR1-dependent phosphorylation of the EGFR in HT-29 cells (Fig. 3A). We checked that AG1478, as a control, also completely inhibited EGF-dependent phosphorylation of the EGFR. It is concluded that activation of PAR1 leads to tyrosine phosphorylation of the EGFR.

Blockade of the EGFR Ligand Binding Domain Inhibits PAR1-Mediated Effects on Cell Proliferation and ERK1/2 Phosphorylation

To determine the mechanism by which EGFR is activated following the challenge of HT-29 cells with PAR1 agonists, we designed experiments to block the binding of EGFR ligands to the extracellular domain of the EGFR. We used a monoclonal antibody directed against the extracellular portion of the EGFR. To avoid the bias of the proteolytic activity of thrombin on the EGFR antibodies, we did these experiments only with AP1 and not with thrombin. As shown in Fig. 4A, preincubation of HT-29 cells with the EGFR blocking antibody significantly reduced PAR1-induced cell proliferation. In consonance with these data, pretreating the cells with this antibody also inhibited AP1-stimulated ERK1/2 phosphorylation (Fig. 4B). To test the efficiency of the antibody, we showed that the anti-EGFR also inhibited EGF-induced ERK1/2 phosphorylation and cell proliferation (Fig. 4). These results provide strong evidence that PAR1-induced cell proliferation and signal transduction involve the binding of an EGFR ligand to the EGFR.
A Neutralizing Antibody for TGF-α Blocked PAR1-Induced Cell Proliferation

The above-described data showed that inhibition of EGFR phosphorylation and blockade of EGFR ligand binding domain inhibit PAR1-induced ERK1/2 activation and subsequent cell proliferation. We next investigated whether EGFR transactivation on activation of PAR1 is mediated by TGF-α release, the most abundant member of the EGFR family ligands found in colon carcinomas (10). We first analyzed the involvement of TGF-α by blocking its biological activity with neutralizing antibodies. As shown in Fig. 5A, treatment of HT-29 cells with TGF-α neutralizing antibodies strongly inhibited AP1-induced cell proliferation. The TGF-α antibodies also had a small effect on control cells, suggesting that small amounts of TGF-α are constitutively secreted in the absence of PAR1 agonists. Furthermore, we investigated whether AP1 causes the release of TGF-α from HT-29 cells. Results showed that AP1 significantly enhanced TGF-α release as compared with control cells (Fig. 5B). Because of the rapid consumption of TGF-α by the EGFR (11), to prevent the binding of released TGF-α to EGFR prior to stimulation with AP1, we preincubated HT-29 cells with EGFR binding site antibodies (12). As shown in Fig. 5A, treatment of HT-29 cells with TGF-α neutralizing antibodies strongly inhibited AP1-induced cell proliferation. The TGF-α antibodies also had a small effect on control cells, suggesting that small amounts of TGF-α are constitutively secreted in the absence of PAR1 agonists.

We also investigated the effect of PAR1 activation on pro-TGF-α content in HT-29-treated cell lysates. No differences in pro-TGF-α content has been detected between control and AP1-stimulated cells (data not shown). These findings suggest that PAR1 transactivates the EGFR by causing the extracellular release of TGF-α but does not alter the cell content of TGF-α at least in our experimental conditions.

FIGURE 2. Effect of the EGFR tyrosine kinase inhibitors AG1478 or PD168393 on PAR1 agonist-induced HT-29 cell proliferation. Cells grown in serum-free medium were treated for 60 minutes with vehicle, AG1478 (3 μM), or PD168393 (2 μM) and PAR1 agonists or EGF were added in the medium. A. Cells were stimulated without or with thrombin (10 nmol/L), AP1 (100 μM/L), or EGF (6 nmol/L) in the presence or absence of AG1478. B. Cells were stimulated without or with thrombin (10 nmol/L), AP1 (100 μM/L), or EGF (6 nmol/L) in the presence or absence of PD168393. After 96 hours, cells from triplicate wells were counted for each condition. Columns, means; bars, SE. ***, P < 0.0001, significant reduction by inhibitors in EGF, thrombin, or AP1-stimulated cell proliferation.

FIGURE 3. Effect of the EGFR tyrosine kinase inhibitor AG1478 on PAR1 agonist-induced EGFR phosphorylation in HT-29 cells. Cells grown in serum-free medium were stimulated without or with PAR1 agonists or EGF in the absence or presence of AG1478. A. Cells were stimulated for 5 minutes with thrombin (10 nmol/L), AP1 (100 μM/L), or EGF (6 nmol/L). B. Cells were pretreated with AG1478 (3 μM) for 60 minutes prior to 5 minutes of stimulation with AP1 (100 μM/L), thrombin (10 nmol/L), or EGF (6 nmol/L). Cell lysates were tested for EGFR phosphorylation. The EGFR was immunoprecipitated (IP) with a polyclonal anti-EGFR and immunoprecipitated proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose. Phosphorylated EGFRs were detected by immunoblot (IB) with the anti-phosphotyrosine monoclonal antibody. EGFR phosphorylation after EGF treatment was tested as a control. EGFR total levels after various treatments were assessed by reprobing the blots with anti-EGFR polyclonal antibody. EGFR protein seems to be reduced in EGF-stimulated samples. This may be an artifact due to the immunoblotting steps because EGFR is detected at similar levels when the immunoblot was directly probed with the anti-EGFR after immunoprecipitation (data not shown). It is suggested that the high signal with the anti-phosphotyrosine antibody prevents subsequent anti-EGFR antibody binding to the EGFR epitope as observed previously (56). Representative immunoblots of three experiments.
A representative immunoblot from two experiments reprobed with anti-ERK1/2 to verify equal protein loading between lanes. The blot was subsequently stripped and re-probed with anti-phospho-ERK1/2. The immunoblot with anti-ERK1/2 and anti-phospho-ERK1/2 antibodies. Cell lysates were directly analyzed for ERK1/2 phosphorylation. Cells grown in serum-free medium were treated for 2 hours without (Control) or with EGFR binding domain antibodies (10 μg/mL). A. Cells were incubated without or with AP1 (100 nmol/L) or EGF (6 nmol/L). After 96 hours, cells from triplicate wells were counted for each condition. Columns, means; bars, SE. ***, P < 0.0001, significant reduction by anti-EGFR in AP1-stimulated or EGF-stimulated cell proliferation. B. Cells were stimulated for 5 minutes with AP1 (100 μmol/L) or EGF (6 nmol/L) in the absence or the presence of the anti-EGFR blocking antibodies. Cell lysates were directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2. The blot was subsequently stripped and re-probed with anti-ERK1/2 to verify equal protein loading between lanes. A representative immunoblot from two experiments.

**FIGURE 4.** Effect of blocking antibodies against the extracellular binding domain of EGFR on PAR1 agonist–induced cell proliferation and ERK1/2 phosphorylation. Cells grown in serum-free medium were treated for 2 hours without (Control) or with EGFR binding domain antibodies (10 μg/mL). A. Cells were incubated without or with AP1 (100 μmol/L) or EGF (6 nmol/L). After 96 hours, cells from triplicate wells were counted for each condition. Columns, means; bars, SE. ***, P < 0.0001, significant reduction by anti-EGFR in AP1-stimulated or EGF-stimulated cell proliferation. B. Cells were stimulated for 5 minutes with AP1 (100 μmol/L) or EGF (6 nmol/L) in the absence or the presence of the anti-EGFR blocking antibodies. Cell lysates were directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2. The blot was subsequently stripped and re-probed with anti-ERK1/2 to verify equal protein loading between lanes. A representative immunoblot from two experiments.

**PAR1-Mediated ERK1/2 Phosphorylation and Cell Proliferation Involve MMPs**

A mechanism whereby the TGF-α ligand is released is thought to be the shedding of membrane-bound precursor by MMPs (13). Therefore, we tested the effect of the general MMP inhibitor batimastat in our model. As shown in Fig. 6, batimastat abrogated cell proliferation induced by AP1 or thrombin. In contrast, batimastat had no effect on exogenous EGF-induced or TGF-α-induced cell proliferation (Fig. 6A). Batimastat was also tested on ERK1/2 phosphorylation induced by PAR1 agonists (Fig. 6B). The MMPs inhibitor dramatically decreased ERK1/2 phosphorylation caused by PAR1 agonists (thrombin or AP1). As expected, batimastat had no effect on EGF-induced ERK1/2 phosphorylation (Fig. 6B). These studies suggest that EGFR transactivation induced by PAR1 agonists involves MMP-mediated EGFR ligand cleavage and correlates with our previous experiments, which showed a reduction in API-induced cell number in the presence of the TGF-α blocking antibody.

**Effect of Src Inhibitor on PAR1 Agonist–Induced EGFR Phosphorylation, ERK1/2 Activation, and Subsequent Cell Proliferation**

In addition to EGFR activation, other tyrosine kinases such as Src can be activated following stimulation of GPCRs (14). To examine the involvement of Src in PAR1-mediated responses in HT-29 cells, we used the Src tyrosine kinase inhibitor PP2. As shown in Fig. 7, pretreatment of HT-29 cells with PP2 (10 μmol/L) did not significantly reduce PAR1 agonist–induced EGFR phosphorylation (Fig. 7A). However, Src inhibition with PP2 significantly blocked ERK1/2 phosphorylation induced by PAR1 agonists in HT-29 cells (Fig. 7B). In concordance with the mitogen-activated protein kinase effect, HT-29 cell proliferation induced by API or thrombin was dramatically decreased in the presence of PP2 (5 μmol/L; Fig. 7C). In contrast, PP2 had only a slight effect, if any, on exogenous EGF-induced EGFR phosphorylation, ERK1/2 phosphorylation, and cell proliferation (Fig. 7). In these studies, we could not use the inactive analogue of Src kinase inhibitor PP3 because of its known effect on the EGFR tyrosine kinase activity (15).

**Effect of PAR1 Activation on Src Phosphorylation**

The above-described data showed that inhibition of Src abrogated PAR1-induced ERK1/2 activation and subsequent cell proliferation. We investigated the activity of Src on activation of PAR1. Because full catalytic activity of Src requires phosphorylation of Tyr^{418}, we tested the effect of PAR1 activation on Src phosphorylation using the anti-Src[pY418] by Western blot. As shown in Fig. 8, activation of PAR1 with thrombin or AP1 induced a significant phosphorylation of Src. Pretreatment of HT-29 cells with the Src inhibitor PP2 resulted in a significant reduction of Src phosphorylation. These data support the involvement of Src tyrosine kinase activity in EGFR transactivation by PAR1. Taken together, these results suggest that PAR1 mediates ERK phosphorylation and cell proliferation by EGFR-dependent and Src kinase–dependent pathways.

**Discussion**

In previous studies, we showed that aberrant expression and activation of the thrombin receptor PAR1 induces cell proliferation and ERK1/2 phosphorylation in human colon cancer cells (4). In the present study, we expand on these findings to elucidate the mechanism(s) whereby PAR1 controls colon cancer cell proliferation. We found that PAR1 agonists trans-activate the EGFR through MMP-dependent cleavage and release of TGF-α.

The different steps of the pathway leading to cell proliferation after activation of PAR1 have been analyzed using pharmacologic enzyme inhibitors and neutralizing antibodies. The important finding of our study was to show that trans-activation of EGFR is an essential link between PAR1 activation and colon cancer cell proliferation. Indeed, blockade of EGFR tyrosine kinase activity by AG1478 or PD168393 and blockade of EGFR binding domain by a specific antibody resulted in robust inhibition of both ERK1/2 phosphorylation and cell proliferation. Our present results represent the first...
demonstration of PAR1-mediated EGFR transactivation in cancer cells. Given the ectopic expression of PAR1 in human colon cancer (4), transactivation of the EGFR by PAR1 becomes an important mechanism leading to aberrant colon cancer cell proliferation. Whether this mechanism can be extended to other cancers such as breast carcinoma (1, 2), pancreatic adenocarcinoma (16), prostate cancer (17), or renal carcinoma (18), in which PAR1 has been identified, remains to be established.

EGFR tyrosine kinase inhibition and blockade of extracellular ligand binding domain of the EGFR result in similar ablation of PAR1-mediated HT-29 cell proliferation. This suggests that the binding of an extracellular ligand comes into play on PAR1 activation. Our data show that TGF-α is such as an extracellular EGFR ligand. Indeed, incubating HT-29 cells with TGF-α neutralizing antibodies completely blocks PAR1 agonist–induced cell proliferation. The total inhibition observed with TGF-α neutralizing antibodies also suggests that other EGFR ligands such as HB-EGF or amphiregulin are not involved in EGFR transactivation by PAR1.

The role of MMP-dependent cleavage of membrane spanning proforms of EGFR ligands has been shown in GPCR-mediated EGFR transactivation (19-24). The involvement of MMPs in the EGFR transactivation by PAR1 in HT-29 cells is likely because the MMP inhibitor batimastat blocks the PAR1-mediated ERK1/2 phosphorylation and cell proliferation. Among the diverse MMPs responsible for the release of EGFR ligands (13, 25), tumor necrosis factor-α–converting enzyme (TACE), a member of the transmembrane MMP-disintegrin family, has been reported to be a key enzyme for the release of TGF-α (26, 27). This is consistent with the recently described role of TACE in tumorigenesis (28) and GPCR-induced cell proliferation (29). Although these reports established TACE as a TGF-α sheddase, TACE-deficient cells were shown to retain ability to shed TGF-α probably due to the action of other metalloproteases (30, 31). HT-29 cells express TACE (32), but no direct evidence exists to implicate TACE as the ADAM(s) responsible for pro-TGF-α cleavage by PAR1 agonists. The identification of TGF-α sheddases involved in transactivation of EGFR on activation of PAR1, muscarinic (19) and prostaglandin E2 receptors (20), will be an important step toward understanding the mechanism underlying TGF-α ectodomain release in the gastrointestinal cancer.

Previous reports have implicated Src kinase activity in the cellular responses to transactivated EGFRs (33-36). Our data show that PP2, a Src tyrosine kinase inhibitor, completely inhibited ERK activation and subsequent cell proliferation stimulated by PAR1 agonists by inhibiting Src phosphorylation on its active site. In contrast, PP2 barely affected EGFR autophosphorylation induced by PAR1 agonists or EGF. This suggests that Src acts downstream of EGFR in the PAR1-stimulated pathway leading to cell proliferation and that Src has a permissive role in mediating cell proliferation after EGFR transactivation by PAR1. This permissive role is not observed when EGFR is directly stimulated by EGF. Although the involvement of Src in transactivation of the EGFR by GPCRs has been reported in several cellular models (20, 37-41), depending on the agonists, Src may act upstream and/or downstream of the EGFR (reviewed in refs. 35, 36). The role of Src as a crucial player in the signaling pathway(s) downstream of the EGFR should be considered in future studies.

\[ \text{FIGURE 5. Effect of TGF-α neutralizing antibodies on PAR1 agonist-induced cell proliferation and effect of AP1 on TGF-α secretion in HT-29 cells. Cells grown in serum-free medium were treated for 2 hours without (Control) or with TGF-α neutralizing antibodies (10 µg/mL). A. Cells were stimulated without or with AP1 (100 µmol/L). After 96 hours, cells from triplicate wells were counted for each condition of treatment. *, } P < 0.05; **, } P < 0.001. B. Cells were preincubated without or with an EGFR binding domain antibody (10 µg/mL) for 2 hours prior to stimulation without or with AP1 (100 µmol/L). Bathing medium was collected and analyzed for TGF-α contents using an ELISA kit. Columns, means; bars, SE. There was a significant increase in TGF-α release in AP1-stimulated cells (n = 4) versus unstimulated cells (n = 4). ###, } P < 0.002. In addition, in the presence of anti-EGFR, there was also a significant increase in AP1-stimulated cells (n = 6) as compared with unstimulated cells (n = 6), *, } P < 0.05. \]
Although the role of thrombin in promoting cell invasion has been reported in many tumors (reviewed in ref. 42), its role in colon cancer proliferation was shown only recently (4). In this context, it is worth noting that a direct association between the thrombin pathway and malignancy is very long known (43). Several observations now underline the relationship between thrombosis and colon cancer. First, clinical observations indicate that the coagulation system is activated in patients with colon cancer (44, 45). This activation of blood coagulation leads to generation of thrombin (46). Second, elevation of the levels of thrombin/antithrombin complexes as well as thrombin fragments has been found in the blood of patients with colon cancer (44), particularly in the draining veins of colon tumors (47). Third, it has been shown clearly that colon cancer cells aggregate platelets by generation of thrombin activity (48, 49). Together with the aberrant expression of PAR1 (4) in human colon cancer cells, these studies suggest that thrombin is involved in autocrine/paracrine loops within the colonic tumor resulting in tumor progression. In this context, thrombin-targeted, anticoagulant strategies designed to affect both the prothrombotic properties of tumors and their growth have been evaluated in several preclinical and clinical studies (42).

FIGURE 6. Effect of the MMP inhibitor batimastat on PAR1 agonist–induced HT-29 cell proliferation and ERK1/2 phosphorylation. Cells grown in serum-free medium were treated for 30 minutes with vehicle or batimastat (5 μmol/L). A. Cells were incubated without (Control) or with thrombin (10 nmol/L), AP1 (100 μmol/L), EGF (6 nmol/L), or TGF-α (6 nmol/L). After 96 hours, cells from triplicate wells were counted for each condition. Columns, means; bars, SE. *, P < 0.05; **, P < 0.001, significant reduction by batimastat in thrombin-, AP1-, EGF-, or TGF-α-stimulated cell proliferation. ###, P < 0.0001, differences between EGF and TGF-α versus control batimastat-treated cells. B. Cells were stimulated without or with thrombin (10 nmol/L), AP1 (100 μmol/L), or EGF (6 nmol/L). Cell lysates were directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2 antibodies. Blots were subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading between lanes. A representative immunoblot from two experiments.

FIGURE 7. Effect of the Src tyrosine kinase inhibitor PP2 on PAR1 agonist–induced EGFR phosphorylation, ERK1/2 activation, and HT-29 cell proliferation. Cells grown in serum-free medium were treated for 60 minutes with vehicle or PP2 (10 μmol/L) prior to stimulation with PAR1 agonists or EGF. A. Lysates from cells stimulated without (Control) or with thrombin (10 nmol/L), AP1 (100 μmol/L), or EGF (6 nmol/L) were tested for EGFR phosphorylation. B. Lysates from cells stimulated as in A were directly analyzed by Western blot for ERK1/2 phosphorylation with anti-phospho-ERK1/2 antibodies. Total levels of EGFR and ERK1/2 after various treatments were assessed by reprobing the blots in A and B with anti-EGFR and with anti-ERK1/2 antibodies, respectively. C. Cells were pretreated for 60 minutes with vehicle or PP2 (5 μmol/L) and stimulated without (control) or with thrombin (10 nmol/L), AP1 (100 μmol/L), or EGF (6 nmol/L). After 96 hours, cells from triplicate wells were counted for each condition. Columns, means; bars, SE. *, P < 0.05; **, P < 0.001; ***, P < 0.0001, significant reduction by PP2 in control or thrombin-, AP1-, or EGF-stimulated cell proliferation.

However, there is still no convincing evidence that this approach can improve survival in cancer. Our present findings identifying the EGFR transactivation as a central signaling element for thrombin-mediated colon cancer cell proliferation suggest that EGFR is a key therapeutic target even for blocking the thrombin-induced colon cancer cell growth. This is in line with several other observations supporting a central role of EGFR in mediating colon cancer progression: (a) ligands for two other GPCRs, the prostaglandin E2 receptor (20) and the M3 muscarinic receptor (50), also lead to colon cancer cell proliferation via EGFR transactivation; (b) up-regulation of the EGFRs and their ligands is frequent in colorectal tumors.
Experiments have a basal Src phosphorylation. A representative immunoblot from two experiments by reprobing the blot with anti-Src antibodies. Note that HT-29 cells have a basal Src phosphorylation. A representative immunoblot from two experiments.

(10, 51); and (c) inhibition of EGFR signaling pathway impairs colonic tumor cell proliferation (52, 53). These findings have potentially important implications regarding blocking EGFR in colon cancer, which are still under way in clinical trials (54).

In summary, our data show that the serine protease thrombin, acting at PAR1, triggers mitogenic signaling in colon cancer cells via EGFR transactivation. This can explain a mechanism by which aberrant expression and activation of the thrombin receptor PAR1 play an important role in the development of colon cancer.

Materials and Methods

Reagents

The reagents were obtained from the following sources: AP1 TFFRLNH2 (Neosystem, Strasbourg, France); highly purified α-thrombin (3,000 units/mg, Kordia Laboratory Supplies, Leiden, Netherlands); the mitogen-activated protein kinase kinase inhibitor PD98059, the EGFR tyrosine kinase–specific inhibitors tyrophostin AG1478 and PD168393, and PP2 (Calbiochem, San Diego, CA); batimastat (BB94, British Biotech, Oxford, United Kingdom); protein G-Sepharose beads (Amersham Pharmacia Biotech, Les Ulis, France). All other chemicals were from Interchim (Asnière, France).

Antibodies were purchased from the following vendors: phosphospecific antibodies to ERK1/2 (New England Biolabs, Beverly, MA); polyclonal anti-ERK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-EGFR used for immunoprecipitation and immunoblotting of EGFR (NeoMarkers, Fremont, CA); rabbit polyclonal anti-Src[pY418] (BioSource International, Camarillo, CA); monoclonal neutralizing anti-EGFR, monoclonal anti-Src and monoclonal anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY); monoclonal neutralizing anti-TGF-α (Oncogene, Boston, MA). ELISA kits for TGF-α measurement were from R&D Systems (Minneapolis, MN) and Oncogene Science (Cambridge, MA).

Cell Culture

HT-29 cells were routinely cultured in 25 cm² plastic flasks (Costar, Cambridge, MA) and maintained at 37°C in humidified atmosphere of 5% CO2/air in DMEM (Life Technologies, Grand Island, NY) containing glucose (4.5 g/L) supplemented with 10% inactivated FCS as described previously (55).

Cell Proliferation Assay

Determination of cellular proliferation was accomplished by direct cell count. Cells (5,000 per well) were seeded in 96 cluster wells (Costar) in the appropriate medium and allowed to attach for 3 days. They were starved in serum-free medium for 48 hours. Then, fresh serum-free medium (200 μL) with or without PAR1 agonists (10 nmol/L thrombin or 100 μmol/L AP1) or EGF (6 nmol/L) was added every other day for 4 days.

In some experiments, HT-29 cells were preincubated for 60 minutes with AG1478 (3 μmol/L), PD168393 (2 μmol/L), or PP2 (5 μmol/L), 30 minutes with batimastat (5 μmol/L), or 2 hours with the blocking antibodies for EGFR or TGF-α prior to cell stimulation with the PAR1 activators. After 96 hours of culture, cells were detached from triplicate wells by trypsin/EDTA and counted in a hemacytometer. Cell death was evaluated with trypan blue and the staining of cells treated with the indicated inhibitors revealed 99.9% viability, suggesting that these concentrations are not toxic to HT-29 cells.

Mitogen-Activated Protein Kinase and Western Blots

For ERK1/2 phosphorylation, cells were grown in six cluster wells (Costar) at 70% confluency and serum deprived for 48 hours prior to their activation with thrombin (10 nmol/L) or AP1 (100 μmol/L) for 5 minutes. In some experiments, prior to the stimulation with the PAR1 agonists, cells were treated with PD98059 (25 μmol/L), AG1478 (3 μmol/L), or PP2 (10 μmol/L) for 1 hour or blocking antibodies for 2 hours. Cells were lysed with radioimmunoprecipitation assay buffer [PBS, 1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS] containing protease inhibitor cocktail and Na3VO4 (1 mmol/L) for 30 minutes at 4°C and lysates were centrifuged at 12,000 × g for 15 minutes. Equal amounts of extracts (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated first with TBS buffer (20 mmol/L Tris, 500 mmol/L NaCl) containing 5% (w/v) low fat milk and 0.1% (v/v) Tween 20 and with phosphospecific antibodies to ERK1/2 diluted 1:2,000 overnight at 4°C that react with the activated, dual threonine and tyrosine phosphorylated forms of p42/p44-mitogen-activated protein kinase. Then, blots were washed and incubated with the anti-iG peroxidase–linked secondary antibody for 1 hour at room temperature before detection using chemiluminescence detection kit (NEN Life Science, Paris, France) and exposure to X-ray films. The same membrane was reprobed with a polyclonal anti-ERK1/2 antibody (diluted 1:1,000), which recognizes ERK1/2 regardless of its phosphorylation status, and served as loading control.

EGFR Immunoprecipitation Assay and Western Blot

HT-29 cells were grown in 100 mm dishes at 70% confluency and serum starved for 48 hours. Cells were treated...
with PAR1 agonists (10 mmol/L thrombin or 100 μmol/L API) for 5 minutes at 37°C before being washed with cold PBS and lysed for 45 minutes on ice in the radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% (v/v) NP40, 0.25% (v/v) sodium deoxycholate, 1 mmol/L EGTA, 1 mmol/L Na₂VO₄, 1 mmol/L NaF, proteases inhibitors]. Cell lysates were cleared by spinning at 12,000 × g for 10 minutes. EGFRs were precipitated overnight at 4°C from lysates with a polyclonal anti-EGFR antibody coupled to protein G-Sepharose beads. The precipitates were separated on 7.5% SDS-PAGE and transferred to nitrocellulose. Phosphorylated EGFRs were detected by Western blot with the anti-phosphotyrosine monoclonal antibody 4G10. The total quantities of EGFR were detected by Western blot with a polyclonal antibody against EGFR (56). In some experiments, prior to the stimulation with the ligands, cells were pretreated with AG1478 (3 μmol/L) or PP2 (10 μmol/L) for 1 hour and stimulated with PAR1 agonists or EGF for 5 minutes.

**Src Kinase and Western Blots**

For Src phosphorylation, cells were lysed in a radioimmunoprecipitation assay buffer as for the EGFR immunoprecipitation. Equal amounts of extracts (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated first with TBS buffer (Tris 20 mmol/L, NaCl 150 mmol/L) containing 5% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20 and overnight at 4°C with anti-Src[pY418] [diluted 1:1,000 in 5% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20] that recognizes the active catalytic form of Src. Then, blots were washed and incubated with the anti-IgG peroxidase–linked secondary antibody as described above. The same membrane was reprobed with a monoclonal anti-Src antibody (diluted 1:1,000), which recognizes p60 Src for equal loading.

**TGF-α ELISA**

HT-29 cells were seeded at 5,000 cells/well in 96 cluster wells (Costar) or 10⁴ cells/well in 12 cluster wells and allowed to attach for 3 days. They were starved in serum-free medium for 48 hours. To prevent the binding of released TGF-α to EGFR, we pretreated cells with the anti-EGFR binding domain antibody (10 μg/mL) for 2 hours prior to cell stimulation with PAR1 agonists. It was hypothesized [19] that this pretreatment increases the measurement of TGF-α in cell culture supernatants. Cells were counted and the conditioned medium was collected for measurement of TGF-α. Lysates from pretreated HT-29 cells were obtained using the CytoBuster buffer (Novagen, Inc., Madison, WI). ELISA for TGF-α was done using commercially available kits according to the manufacturer’s instructions. The TGF-α antibody in the ELISA kit recognizes the mature form and the pro-form of TGF-α.

**Statistical Analysis**

All results are expressed as means ± SE for a series of experiments. Differences between data were tested by Student’s t test for unpaired data. P < 0.05 was considered statistically significant.

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**References**


Activation of Proteinase-Activated Receptor 1 Promotes Human Colon Cancer Cell Proliferation Through Epidermal Growth Factor Receptor Transactivation

Dalila Darmoul, Valérie Gratio, Hélène Devaud, et al.


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