Prostaglandin E2 Enhances Pancreatic Cancer Invasiveness through an Ets-1–Dependent Induction of Matrix Metalloproteinase-2

Hiromichi Ito, Mark Duxbury, Eric Benoît, Thomas E. Clancy, Michael J. Zinner, Stanley W. Ashley, and Edward E. Whang

Department of Surgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Accumulating evidence suggests an important role for cyclooxygenase-2 (COX-2) in the pathogenesis of a wide range of malignancies. Here we tested the hypothesis that the COX-2 product prostaglandin E2 (PGE2) increases cellular invasive potential by inducing matrix metalloproteinase-2 (MMP-2) expression and activity through an extracellular signal-regulated kinase (ERK)/Ets-1–dependent mechanism in pancreatic cancer. PANC-1 and MIAPaCa-2 pancreatic cancer cells were treated with PGE2 or rofecoxib, a selective COX-2 inhibitor. MMP-2 expression and activity were assayed using Western blot analysis and zymography, respectively. MMP-2 promoter activity was analyzed with a luciferase-based assay. Ets-1 activity was analyzed using gel shift assay. Ets-1 expression was specifically silenced using RNA interference. Cellular invasive and migratory potentials were determined using a Boyden chamber assay with or without Matrigel, respectively. Exogenous PGE2 induced MMP-2 expression and activity and increased ERK1/2 phosphorylation, Ets-1 binding activity, and MMP-2 promoter activity. PGE2 also increased cellular migratory and invasive potentials. The mitogen-activated protein kinase kinase inhibitor PD98059 and Ets-1 silencing each abolished PGE2-induced increases in MMP-2 expression. PD98059 and Ets-1 silencing each abrogated the effect of PGE2 on cellular invasive potential but not on cellular migratory potential. Rofecoxib suppressed MMP-2 expression and activity, Ets-1 binding activity, MMP-2 promoter activity, and cellular migratory and invasive potentials. These results suggest that PGE2 mediates pancreatic cancer cellular invasiveness through an ERK/Ets-1–dependent induction of MMP-2 expression and activity. They also suggest that COX-2 inhibition may represent a strategy to inhibit invasive potential in pancreatic cancer.

INTRODUCTION

Cyclooxygenase-2 (COX-2) overexpression has been reported to be present in 74% to 100% of human pancreatic cancer specimens (1–3). COX-2 is the inducible isoform of COX, the enzyme catalyzing the rate-limiting step in the metabolic pathway that transforms arachidonic acid into the prostaglandins (PGs) and thromboxanes, whereas COX-1 is constitutively expressed in most tissues (4). Among the products of COX-2, prostaglandin E2 (PGE2), in particular, may play a biologically important role in cancer pathogenesis. Many cancers that overexpress COX-2 have high intratumoral levels of PGE2 (5–7), and PGE2 recently was shown to up-regulate the invasive potential of colorectal carcinoma cells (8).

We have reported previously that matrix metalloproteinase-2 (MMP-2) activity is an important determinant of pancreatic cancer cellular invasive potential (9). Ets-1 is a member of the Ets family of transcription factors that share a conserved Ets domain (10). It has been reported that Ets-1 is overexpressed in a variety of human malignancies, including cancers of the ovary (11), breast (12), colon and rectum (13), and pancreas (14). Because of its roles in the transcriptional regulation of MMPs, including MMP-2 (15, 16), Ets-1 is a candidate mediator of cancer invasion and metastasis.

In this study, we tested the hypothesis that PGE2 increases pancreatic cancer cellular invasive potential through an Ets-1–dependent induction of MMP-2 expression and activity. We also tested the efficacy of COX-2 inhibition in inhibiting Ets-1–dependent MMP-2 induction and cellular invasive potential.
conditioned media were harvested for zymography. Zymography was carried out as described previously (17). In brief, 25 μL of the conditioned medium for each sample were subjected to 10% SDS/PAGE with 1 mg/mL gelatin incorporated into the gel mixture. Following the electrophoresis, the gels were soaked in 2.5% Triton X to remove SDS, rinsed with 10 mmol/L Tris (pH 8.0), and transferred to a bath containing 50 mmol/L Tris (pH 8.0), 5 mmol/L CaCl₂, and 2 μmol/L ZnCl₂ at 37°C for 17 hours. The gels then were stained with 0.1% Coomasie blue in 45% methanol and 10% acetic acid. Experiments were repeated three times.

Cloning of MMP-2 Promoter. The human MMP-2 promoter was cloned from human placental DNA by PCR using primers (5'-CACACCCAGCAAGCGCCCT-3' and 5'-AAGCGCCAGATCCGACCTG-3') as described previously (18). The PCR product (the 1716-bp DNA fragment covering positions -1659 to +57 relative to the MMP-2 transcription initiation site) was cloned into the pGL3 vector (Promega, Madison, WI). A deletion mutant MMP-2 promoter (1081-bp fragment covering positions -1024 to +57 relative to the MMP-2 transcription initiation site) was made from the full-length MMP-2 promoter; it was digested by EcoRI, filled with Klenow fragment of DNA polymerase I, and religated. The vectors containing the inserts are designated pGL3-MMP2 (containing full-length promoter) and pGL3-MMP2Δ (containing deletion mutant promoter); sequencing was performed to confirm correct insert orientation.

Luciferase Assay. The luciferase assay was conducted as described previously (19). Empty pGL3 vector (pGL3ce) was used for control transfections. Cells (4 × 10⁵ cells/well) were plated onto wells of 12-well cell culture dishes and cultured for 12 hours in serum-containing medium. One microgram of pGL3-MMP2, pGL3-MMP2Δ, or pGL3ce was cotransfected with 0.1 μg of pRL-CMV vector (Promega), which contains a cytomegalovirus promoter upstream of a renilla luciferase gene. Immediately afterward, cells were treated with either PGE₂ (10 μmol/L) or RFX (5 μmol/L). After 24 hours, luciferase activities in lysates of the transfected cells were measured, according to the manufacturer’s recommended protocol (Dual-Luciferase Reporter Assay System; Promega). The activities of renilla luciferase were used to normalize any variations in transfection efficiency. The promoter activities of each plasmid construct were calculated as the firefly-renilla luciferase activity ratios. Measurements were performed three times per sample, and results are shown as mean ± SD of three independent experiments.

Nuclear Extract Preparation. Nuclear extracts were obtained using a modification of a previously described method (20). Harvested cells were lysed with buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1.5 mmol/L MgCl₂, 0.2% NP40, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride) followed by vortexing to shear the cytoplasmic membranes. Nuclear fractions were then collected by centrifugation at 3000 rpm for 30 seconds at 4°C in a microcentrifuge. Nuclear proteins were extracted with high-salt buffer C (20 mmol/L HEPES, 1.5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 420 mmol/L NaCl, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride). Total nuclear protein was determined by BCA assay.

Electrophoretic Mobility Shift Analysis. Ets-1 and Oct-1 transcription factor activities were assessed by electrophoretic mobility shift analysis (EMSA) using double-stranded oligonucleotides corresponding to the Ets-1 or Oct-1 consensus sequences (Ets-1, 5'-GATCGTCGCAATGCATCTAGGAAGAAGTTGAG-3'; OCT-1, 5'-GTGTGGATGCAGAATCCTAGAAG-3'; Santa Cruz Biotechnology), respectively. Probes were end-labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Labeled probes were purified by Nick column (Amersham Biosciences, Piscataway, NJ). Nuclear protein (5 μg) was incubated with labeled probe in a binding buffer containing 2 μg of poly(dI:dC) (Amersham Biosciences), 20 mmol/L HEPES (pH 7.9), 10% glycerol, 1 mmol/L EDTA, 5 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 mmol/L NaCl for 20 minutes at room temperature. Protein-DNA complexes were resolved by electrophoresis through a 6% polyacrylamide gel and were electrophotoretically transferred to nylon membranes (Invitrogen). The membranes were then subjected to autoradiography. Band specificities were determined in competition experiments using a 100-fold molar excess of unlabeled nucleotide containing either the Ets-1 consensus sequence or a mutated sequence (Ets-1, 5'-GATCTGGAGCAAGAAGTTGAG-3'; Santa Cruz Biotechnology) that was added to nuclear extracts for 10 minutes before addition of radiolabeled probe. Ets-1 supershift was performed using an anti-Ets-1 monoclonal antibody. The antibody (1 μg/mL final concentration) was added after the oligonucleotide had reacted for 20 minutes with the nuclear extract and allowed to react for 45 minutes at room temperature before electrophoresis.

Small Interfering RNA Synthesis. Small interfering RNAs (siRNAs) were synthesized and high-performance purified (Qiagen, Valencia, CA). Ets-1 sense (ACUUUCCUACCAACCGUAC-3') and antisense (GUAAGGUAGGUAGCAAGAG-3') siRNAs and control siRNAs (sense, AGGAGAUUUCGAGCCGU-3'; antisense, UCGCAUUAAUCUCCU-3') were dissolved in buffer (100 mmol/L potassium acetate, 30 mmol/L HEPES-potassium hydroxide, and 2 mmol/L magnesium acetate (pH 7.4)) to a final concentration of 20 μmol/L, heated to 90°C for 60 seconds, and incubated at 37°C for 60 minutes before use.

Small Interfering RNA Transfection. Cells (5 × 10⁵) were plated onto wells of six-well culture plates and allowed to adhere for 24 hours. Eight microliters siPORT Amine transfection reagent (Ambion Inc., Austin, TX) per well were added to serum-free DMEM for a final complexing volume of 200 μL, vortexed, and incubated at room temperature for 20 minutes. Two microliters of siRNA solution were added to the diluted siPORT Amine transfection reagent, gently mixed, and incubated at room temperature for 15 minutes. The transfection agent/siRNA complex was added into the wells containing 800 μL DMEM with 10% FBS and incubated in normal cell culture conditions for 6 hours, after which time 1 mL DMEM with 10% FBS was added. All of the assays were performed after 48 hours.

Invasion and Migration Assays. The BD Biocoat Matrigel invasion chamber (BD Biosciences, San Jose, CA) was used according to the manufacturer’s instructions. In brief, 2.5 × 10⁵ cells in serum-free media were seeded onto the Matrigel-coated filters, and in the lower wells 5% FBS was added as the chemoattractant. After 24 hours incubation, the filters were stained with Diff-Quik kit (BD Biosciences), and the number of cells that had penetrated through the filter was counted under magnification (randomly selected high-power fields).
power fields). The counting was performed for three fields in each sample. In additional invasion experiments, anti–MMP-2 antibody (100 μg/mL or 400 μg/mL) was added to neutralize MMP-2 activity as described previously (21). To assess cellular migratory potential, the protocol described above was used, except that Matrigel was omitted. Experiments were performed in triplicate, and the results were shown as mean ± SD of three independent experiments.

**Statistical Analysis.** Differences between groups were analyzed using two-sided t test and ANOVA with P < 0.05 considered statistically significant. In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD.

**RESULTS**

**COX-2 Expression and the Effect of Rofecoxib on PGE_2 Synthesis.** First, we examined COX-2 expression and endogenous PGE_2 production in the pancreatic cancer cell lines using Western blot analysis and PGE_2 ELISA, respectively. For PANC-1 and MIAPaCa-2 cells, COX-2 expression was confirmed; the selective COX-2 inhibitor RFX (5 μmol/L) reduced PGE_2 production by 61% and 70%, respectively, in these cell lines (P < 0.05; Fig. 1).

**Effects of PGE_2 and Rofecoxib on MMP-2 Expression and Activity.** To determine the effects of PGE_2 on MMP-2 expression and activity, cells were administered exogenous PGE_2 (10 μmol/L). In parallel experiments, RFX (5 μmol/L) was administered to suppress endogenous PGE_2 production. As shown in Fig. 2A, exogenous PGE_2 increased MMP-2 protein expression by 85% and 122% (P < 0.05) in PANC-1 and MIAPaCa-2 cells, respectively. In contrast, RFX reduced MMP-2 expression by 48% and 55% (P < 0.05) in PANC-1 and MIAPaCa-2 cells, respectively. The effects of PGE_2 and RFX on MMP-2 gelatinolytic activity paralleled those on MMP-2 expression in PANC-1 and MIAPaCa-2 cells, as shown on zymography (Fig. 2B).

**Effects of PGE_2 and Rofecoxib on MMP-2 Transcriptional Activity.** Because of the observed alterations in MMP-2 expression induced by PGE_2 and by RFX, we next sought to determine the effects of PGE_2 and RFX on MMP-2 transcriptional activity using a luciferase-based reporter assay. The full-length MMP-2 promoter was incorporated into the pGL3 luciferase vector and transfected into the cells together with the pRL-CMV plasmid. The resulting luciferase activity was measured at 24 hours. As shown in Fig. 3A, PGE_2 increased MMP-2 promoter activities by 81% and 124% (P < 0.05) in PANC-1 and MIAPaCa-2 cells, respectively, whereas RFX reduced MMP-2 promoter activities by 41% and 46% (P < 0.05), respectively, in PANC-1 and MIAPaCa-2 cells. To localize the responsive element in the MMP-2 promoter sequence, the truncated MMP-2 promoter with deletion of the region spanning positions −1659 to −1025 relative to the MMP-2 transcription initiation site was constructed, and the effect of this deletion on PGE_2-induced luciferase activity was analyzed. As shown in Fig. 3B, deletion of aforementioned region reduced baseline promoter activities 39% and 34% in PANC-1 and MIAPaCa-2, respectively (P < 0.05), and abolished the effect of PGE_2 on promoter activity. This result implicates the region spanning positions −1659 to −1025 of the MMP-2 promoter in PGE_2-mediated induction of MMP-2 transcription.

**Effect of PGE_2 on Transcription Factor Ets-1 Binding Activity.** Ets-1 is a known transcriptional regulator of MMP-2 expression (15, 22), which binds to the region spanning −1255 to −1248 of the
Immediately after transfection, cells were treated with PGE2 (10 μmol/L), and luciferase activity in cell lysates was assayed 24 hours subsequently. The induced firefly luciferase activity was normalized to the renilla luciferase activity. PGE2 induced ERK1/2 phosphorylation within 30 minutes after its administration (Fig. 6A); PD98059 inhibited PGE2-mediated ERK1/2 phosphorylation (Fig. 6B). We then determined the effect of inhibiting ERK1/2 activation on the PGE2-induced increase in Ets-1 binding activity using EMSA. The induction of Ets-1 binding by PGE2 was abolished by PD98059, as shown in Fig. 6C.

These findings suggest that PGE2-induced Ets-1 binding activity is mediated by ERK signaling.

Effects of PGE2 on Cellular Invasion and Migration. We previously have shown that MMP-2 activity is associated with pancreatic cancer invasive potential (9, 24). Here we tested the effects of PGE2 and RFX on cellular invasive and migratory potentials. PGE2 induced 2.8-fold and 2.9-fold increases in PANC-1 and MIAPaCa-2 cellular invasiveness through Matrigel, respectively, relative to untreated cells (P < 0.05). In contrast, RFX induced 63% and 66% reductions in cellular invasiveness in MIAPaCa-2 and PANC-1, respectively, relative to untreated controls (P < 0.05). PGE2 induced 3.2-fold and 3.7-fold increases in PANC-1 and MIAPaCa-2 cellular migration, respectively (P < 0.05); RFX induced 55% and 58% reductions in cellular migration, respectively (Fig. 7A; P < 0.05). Ets-1 silencing reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 30% and 34%, respectively, and abrogated PGE2-mediated increases in cellular invasiveness by 65% and 63%, respectively, relative to PGE2-treated control cells (P < 0.05). Interestingly, Ets-1 silencing altered neither baseline cellular migration nor PGE2-induced increase in cellular migration in PANC-1 and MIAPaCa-2 cells (Fig. 7B). The effects of MAPK kinase inhibition on cellular invasiveness and migration were similar to those of Ets-1 gene silencing. PD98059 (40 μmol/L) reduced baseline PANC-1 and MIAPaCa-2 cellular invasiveness by 40% and 44%, respectively (P < 0.05), and abrogated PGE2-induced increases in cellular invasiveness by 63% and 74%, respectively (P < 0.05), relative to PGE2-treated control cells. There was no significant effect of PD98059 on cellular migration (Fig. 7C).

To examine the relationship between observed alterations in invasiveness and in MMP-2 expression, Matrigel invasion assays were conducted in the presence of MMP-2 neutralizing antibody. PGE2-mediated increases in PANC-1 and MIAPaCa-2 cellular invasiveness through Matrigel were abrogated by anti-MMP-2 antibody but not by control IgG (Fig. 7D). These findings suggest that PGE2-mediated effects on pancreatic cancer cellular invasiveness through Matrigel are at least in part MMP-2 dependent.

**DISCUSSION**

There is much evidence that implicates important roles for COX-2 and PGE2 in carcinogenesis and progression for a wide range of malignancies (3, 25–29), including pancreatic cancer (2, 3). However, there are few data on the downstream targets by which COX-2 and PGE2 mediate these processes. In this study, we...
identified Ets-1 as an important mediator of PGE_2-induced induction of MMP-2 expression. Our study is the first to establish the association between PGE_2 and Ets-1 in cancer; it also provides evidence that COX-2 may mediate cancer invasion by inducing MMP-2.

The potentially important roles of COX-2 and PGE_2 in cancer invasion are supported not only by the observed relationship between COX-2 overexpression and cancer invasion or metastasis but also by other in vitro data. Tsujii et al. (30) showed that forcibly overexpressing COX-2 increases MMP-2 expression/activation and cellular invasiveness in colon cancer cells. Administration of COX-2 inhibitors also has been reported to suppress MMP-2 expression and invasiveness in cells derived from a range of cancers, including those of the colon and rectum (31, 32), breast (33), lung (34), and pancreas (35).

Our study builds on these previously reported findings by revealing a potential mechanism by which COX-2–derived PGE_2 mediates MMP-2 expression and cellular invasiveness. We hypothesized that Ets-1 might play a role in PGE_2-mediated MMP-2 induction. Ets-1 has been reported to be overexpressed in variety of malignancies, including pancreatic cancers (14), and to regulate the transcription of many MMPs, including MMP-2 (15, 22, 36, 37). A recently reported study localized an Ets-1 binding site to the MMP-2 promoter (18). Our findings that PGE_2 induces Ets-1 expression and invasiveness in cells derived from a range of cancers, including those of the colon and rectum (31, 32), breast (33), lung (34), and pancreas (35).

Our study builds on these previously reported findings by revealing a potential mechanism by which COX-2–derived PGE_2 mediates MMP-2 expression and cellular invasiveness. We hypothesized that Ets-1 might play a role in PGE_2-mediated MMP-2 induction. Ets-1 has been reported to be overexpressed in a variety of malignancies, including pancreatic cancers (14), and to regulate the transcription of many MMPs, including MMP-2 (15, 22, 36, 37). A recently reported study localized an Ets-1 binding site to the MMP-2 promoter (18). Our findings that PGE_2 induces Ets-1 expression and invasiveness in cells derived from a range of cancers, including those of the colon and rectum (31, 32), breast (33), lung (34), and pancreas (35).
binding activity and MMP-2 promoter activity and that ETS-1 silencing abolishes the effect of PGE₂ on MMP-2 expression clearly support our hypothesis.

We further explored up-stream regulation of Ets-1 binding activity. PGE₂ previously has been reported to activate MAPK signaling in cancer cell lines (8, 23). Nonsteroidal anti-inflammatory drugs also are reported to suppress ERK1/2 phosphorylation (38). MAPK signaling is reported to enhance Ets-1/Ets-2 binding activity (39). These data, taken together with ours, suggest that Ets-1 binding activity is modified by MAPK signaling provoked by PGE₂. The precise mechanisms by which ERK regulates Ets-1 DNA binding affinity remain to be defined. Some studies suggest that Ets-1 protein is induced by MAPK signaling (40, 41). In our study, although Ets-1 binding activity peaked at 4 hours after administration of PGE₂, no changes in Ets-1 protein expression levels were observed to occur during this period (data not shown). This finding suggests the presence of other mechanisms regulating Ets-1 binding activity. Further studies are required to determine the role of interaction between Erk-1 and these other transcription factors in the context of ERK/Ets-1 signaling.

Our data show that PGE₂ increases not only MMP-2–dependent cellular invasiveness through Matrigel but also cellular migratory motility through phosphatidylinositol 3’-kinase/Akt signaling (8). In our study, neither Ets-1 gene silencing nor MAPK kinase inhibition affected PGE₂-mediated cellular migration. This finding suggests that the effects of PGE₂ on MMP-2 expression and on cellular migration are regulated through separate signaling pathways. They further suggest that PGE₂ may activate multiple signaling pathways that have synergetic effects on cancer invasion.

The relationship between COX-2 expression and cancer invasion has been observed in several human malignancies (25, 46), including pancreatic cancer (1, 2). Our findings provide a mechanistic explanation for these observations. Detailed information on the molecular signaling pathways driving cancer invasion is important for two reasons: First, key components of these signaling pathways represent candidate biomarkers that may predict patient outcomes more reliably than traditional morphologic criteria; and second, understanding these pathways may suggest potential therapeutic targets for inhibiting invasion, a process that is a major contributor to death among patients with cancers such as those of the pancreas.

In summary, our finding indicates that COX-2–derived PGE₂ promotes pancreatic cancer cellular invasive potential through an ERK/Ets-1–dependent induction of MMP-2. This information provides a mechanistic rationale for the observed relationship between COX-2 overexpression and pancreatic cancer progression; targeting this pathway may be a potential therapeutic strategy to inhibit the progression of this highly invasive cancer.
untreated control), and abrogated PGE2-induced increases in invasiveness by 65% and 63%, respectively (\(P < 0.05\), versus control). Right, PGE2-induced increases in cellular migration by 224% and 268% in PANC-1 and MIAPaCa-2 cells, respectively (\(P < 0.05\), versus control), and RFX reduced cellular migration by 55% and 58%, respectively (\(P < 0.05\), versus control). B, the effect of Ets-1 gene silencing. Left, Ets-1 gene silencing reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 30% and 34%, respectively (\(P < 0.05\), versus untransfected control) and abrogated PGE2-induced increases in invasion by 65% and 63%, respectively (\(P < 0.05\), versus PGE2-treated untransfected control cells). Right, Ets-1 gene silencing had no effect on either PANC-1 and MIAPaCa-2 baseline cellular migration or PGE2-induced increases in cellular migration (\(P > 0.05\), versus corresponding control cells that received no PGE2). C, the effect of PD98059. Left, PD98059 (40 \(\mu\)mol/L) reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 40% and 44%, respectively (\(P < 0.05\), versus untreated control), and abrogated PGE2-induced increases in invasion by 63% and 74%, respectively (\(P < 0.05\), versus PGE2-treated controls). Right, PD98059 had no effect on either PANC-1 or MIAPaCa-2 baseline cellular migration or PGE2-induced increases in cellular migration (\(P > 0.05\), versus corresponding control cells that received no PGE2). D, the effect of MMP-2 neutralizing antibody on cellular invasiveness. Cellular invasiveness was assayed in the presence of anti-MMP-2 antibody or control IgG. PGE2-induced increases in cellular invasiveness were abrogated by anti-MMP-2 antibody but not by control IgG (\(P < 0.05\), versus PGE2-treated control).

ACKNOWLEDGMENTS

We thank Jan Rounds for her excellent secretarial assistance.

REFERENCES


Prostaglandin E$_2$ Enhances Pancreatic Cancer Invasiveness through an Ets-1-Dependent Induction of Matrix Metalloproteinase-2

Hiromichi Ito, Mark Duxbury, Eric Benoit, et al.