Focal adhesion kinase: Important to prostaglandin E_2-mediated adhesion, migration and invasion in hepatocellular carcinoma cells

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Abstract. Prostaglandin E_2 has been implicated in cell growth and metastasis in many types of cancers. However, the effects of PGE_2 and its mechanism on cell adhesion, migration, and invasion have not been clarified yet. In this study, we found PGE_2 treatment significantly increased the cell adhesion, migration, and invasion in hepatocellular carcinoma (HCC) cells. In addition, the effects of PGE_2 were found to be associated with focal adhesion kinase (FAK). PGE_2 treatment increased the phosphorylation and synthesis of FAK in a dose-dependent manner. RNA interference targeting FAK suppressed PGE_2-mediated cell adhesion and migration. Furthermore, the downstream proteins of FAK, paxillin and Erk2, were also activated by PGE_2. PGE_2 treatment increased the phosphorylation and synthesis of paxillin in a dose-dependent manner. PGE_2 treatment also induced the phosphorylation of Erk2. PD98059, the specific inhibitor of MEK, suppressed PGE_2-mediated cell adhesion and migration. However, it had no effect on PGE_2-induced activation and synthesis of FAK. These results demonstrated that PGE_2 greatly induced HCC cell adhesion, migration, and invasion by activating FAK/paxillin/Erk pathway.

Introduction

Prostaglandin E_2 (PGE_2) is one of predominant metabolic products of arachidonic acid. Cyclooxygenase (COX) is known as prostaglandin endoperoxide synthase. Cyclooxygenase catalyzes the arachidonic acid to PGH_2. The PGH_2 is then converted into PGE_2, PGI_2, PGD_2, PGF_2, or thromboxane A_2 by each respective PG synthase.

Cyclooxygenase has three isoforms: COX-1, COX-2 and COX-3 (1). COX-1 is expressed constitutively in most tissues, and is responsible for maintaining basic physiologic functions; COX-2 is not constitutively expressed, but is induced by stimuli such as cytokines, hormones, mitogens, and growth factors, which explains its up-regulation in various inflammatory diseases and human cancers (2,3). COX-2 is now proved to be associated with carcinogenesis. COX-2 overexpression leads to increased survival in many cancer types (4-7), such as colorectal, prostatic and bladder cancer. Selective inhibitors of COX-2, e.g. celecoxib, have been shown to suppress tumor cell growth and induce apoptosis (8-11). In previous studies, PGE_2 was found to play a major role in promoting tumor cell growth (5,7,9-11); exogenous PGE_2 mimics the effects of COX-2 overexpression, and celecoxib-induced inhibition of cell growth is partially reversed by PGE_2. PGE_2 was also found to correlate with malignant cell invasion. The PGE_2-mediated migration or invasion is coordinated by a number of proteins, including CD44, EGFR, MMP, and MAPK (12-15).

In our previous studies, we have proved that COX-2 and PGE_2 could promote cell growth and selective COX-2 inhibitor celecoxib could mediate cell apoptosis in hepatocellular carcinoma (HCC) (11,16). However, the effects of PGE_2 and its mechanism on cell adhesion, migration, and invasion have not been clarified in HCC. In recent studies, Integrins are proved to play a central role in the process of cell migration and invasion. They control the cell movement, morphology, cell growth and gene expression by associated with focal adhesion kinase (FAK) and the other downstream signaling proteins (15,17). It was also reported that phosphorylation of FAK and its downstream, paxillin, were required in early phase of breast cancer metastasis (18). Thus, this study analyses the effects of PGE_2 on HCC cell adhesion, migration, invasion, and its relationship with FAK, paxillin and Erk2.

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We demonstrated that PGE_2 significantly increased HCC cell adhesion, migration, and invasion, and that FAK/paxillin/Erk pathway was involved in these processes.

Materials and methods

Materials. Human hepatocellular carcinoma (HCC) cell line HUH-7 was obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (USA). Prostaglandin E_2 (PGE_2) was from Cayman Chemical Co. (USA). Transwell unit (12-well) was from Costar Corning (USA). Matrigel and PD98059 were from Sigma Chemical Co. (USA). Cell counting kit-8 was from Dojindo laboratories (Japan). Lipofectamine™ 2000 was from Invitrogen. Bio-Rad protein assay was from Hercules (CA). Nitrocellulose blotting membranes and electrochemiluminescence (ECL) were from Amersham Biosciences (USA). Anti-FAK antibody (sc-558), anti-Paxillin antibody (sc-5574), anti-Erk2 antibody (sc-154) and anti-phosphorylated Paxillin antibody (sc-14035) were from Santa Cruz (CA). Anti-phosphorylated FAK antibody (No. 611806) was from B&D Transduction Laboratories. Anti-phosphorylated Erk antibody (No. 9106) was from Cell Signaling (USA). Anti-ß-actin antibody was from Sigma Chemical Co.

Methods

Cell culture. The HUH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37˚C with 5% CO_2.

Cell adhesion assays. Cell adhesion assays were performed in 96-well cultured cell cluster. Cells ($5x10^4$) were added to the plates. Pharmacological agents were added at the indicated time. The plates were incubated at 37˚C for 3 h, and then washed 2 times with phosphate-buffered saline (PBS) to remove unattached cells. The attached cells were stained with WST at 37˚C for 1 h. The adherent cells were quantified by the absorbance at 450 nm.

Cell migration assays. Cell migration was assayed in 12-well transwell units. Before the experiment, the lower surfaces of the membranes were coated with gelatin (1%) diluted in PBS. HUH-7 cells ($5x10^4$) were added to the upper chamber, and 1 ml complete DMEM to the lower chamber of the transwell. Pharmacological agents were added at certain time. After incubation at 37˚C for 12 h, the cells were fixed by ethanol, and then stained by 0.1% crystal violet for 30 min at room temperature. After washing the wells with PBS, the cells were removed from upper surface of the membrane by wiping with a moist cotton swab. The cells migrated to lower surfaces of the membranes were photographed under the microscope. Finally, the migrated cells were solubilized with 300 μl of 10% acetic acid, and quantified by the absorbance at 570 nm.

Cell invasion assays. Cell invasion was assayed in 12-well transwell units. Before the experiment, transwell chambers were coated with a layer of 1:10 Matrigel; the lower surfaces of the membranes were coated by gelatin (1%) diluted with PBS. HUH-7 cells ($5x10^4$) were added to the upper chamber and 1 ml complete DMEM added to the lower chamber of the transwell. Pharmacological agents were added when cells were plated. After incubation at 37˚C for 24 h, the cells were fixed by ethanol, and then stained by 0.1% crystal violet for 30 min at room temperature. After washing the wells with

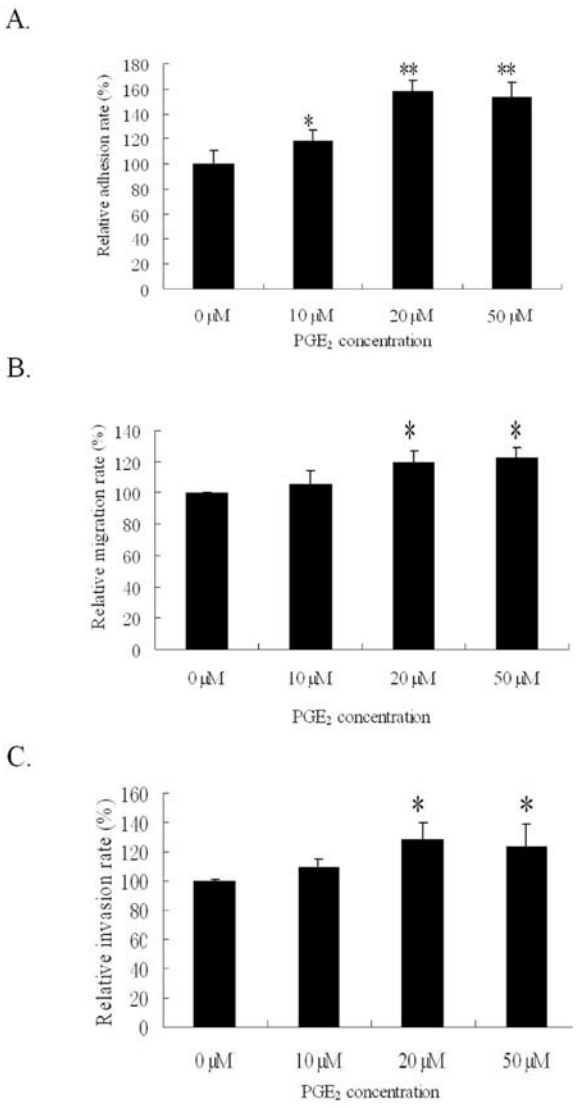

Figure 1. The effects of PGE_2 treatment on the cell adhesion, migration and invasion in HCC cells. (A) Cell adhesion assay. Cell adhesion assay was performed in 96-well plates by incubation for 3 h at 37˚C in serum-free DMEM supplemented with or without PGE_2. The attached cells were stained with WST, and quantified by optical density (OD) with the absorbance at 450 nm. (B) Cell migration assay. HUH-7 cells ($5x10^4$ cells) were placed in the upper chamber of 12-well transwells with or without PGE_2 in serum-free DMEM, and allowed to migrate for 12 h. The cells on the lower surface were fixed by ethanol, and stained by 0.1% crystal violet. The migrated cells were solubilized with 300 μl 10% acetic acid solution, and quantified by the absorbance at 570 nm. (C) Cell invasion assay. The cells were placed in the upper chamber coated with 1:10 Matrigel, and incubated for 24 h. The invasion cells on the lower surface were solubilized with acetic acid solution, and quantified as described above. Results were presented as mean ± SD from three different experiments. *Significant difference at $P<0.05$ when compared to the cells without treatment. **Significant difference at $P<0.01$ when compared to the cells without treatment.

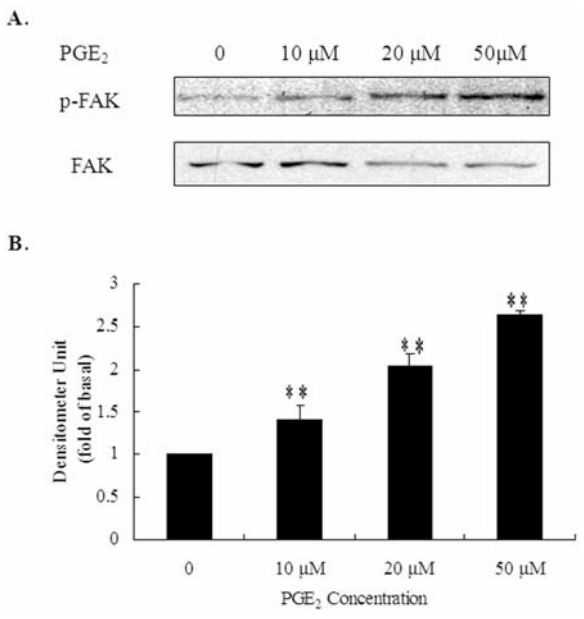

Figure 2. PGE_2 induced FAK phosphorylation at Tyr397 site in HCC cells. (A) HUH-7 cells were cultured, and treated with different concentrations of PGE_2. FAK phosphorylation was increased after the stimulation with different concentrations of PGE_2 for 30 min. Relative levels of phosphorylated FAK and total FAK expression were determined using anti-phospho-FAK and anti-FAK antibodies as described in the Materials and methods. Levels of ß-actin served as a loading control (data not shown). (B) Densitometric quantitation of the above blots. Results were the mean ± SD from three different experiments. **Significant difference at P<0.01 when compared to the cells without treatment.

PBS, the cells were removed from upper surface of the membrane by wiping with a moist cotton swab. The cells invaded to lower surfaces of the membranes were solubilized with 10% acetic acid, and quantified by the absorbance at 570 nm.

Western blotting. Cells were treated with PGE_2 at 37˚C for various times as indicated in the experiments. The cells were collected in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCL, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 100 μg/ml PMSF, and aprotinin) at ice for 30 min. Cell lysates were cleared by centrifugation at $1.2x10^4$ g for 30 min at 4˚C. Total proteins (30 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with antibodies at 4˚C overnight with gentle shaking. The antibodies used were anti-FAK, anti-Paxillin, anti-Erk2, anti-phosphorylated FAK, anti-phosphorylated Paxillin, and anti-phosphorylated Erk antibody. The immunoreactivity was detected with electrochemiluminescence (ECL) and analyzed by Image-J analysis software.

RNA interference. The constructed pRNAT-U6.1 plasmid targeting FAK (psiFAK) was kindly provided by Professor Zichun Hua, Nanjing University, P.R. China (19). HUH-7 cells ($2x10^5$) were seeded into 6-well cultured plate 24 h before the transfection, resulting in a confluence of the cell monolayer of 60-70%. The cells were then cultured by

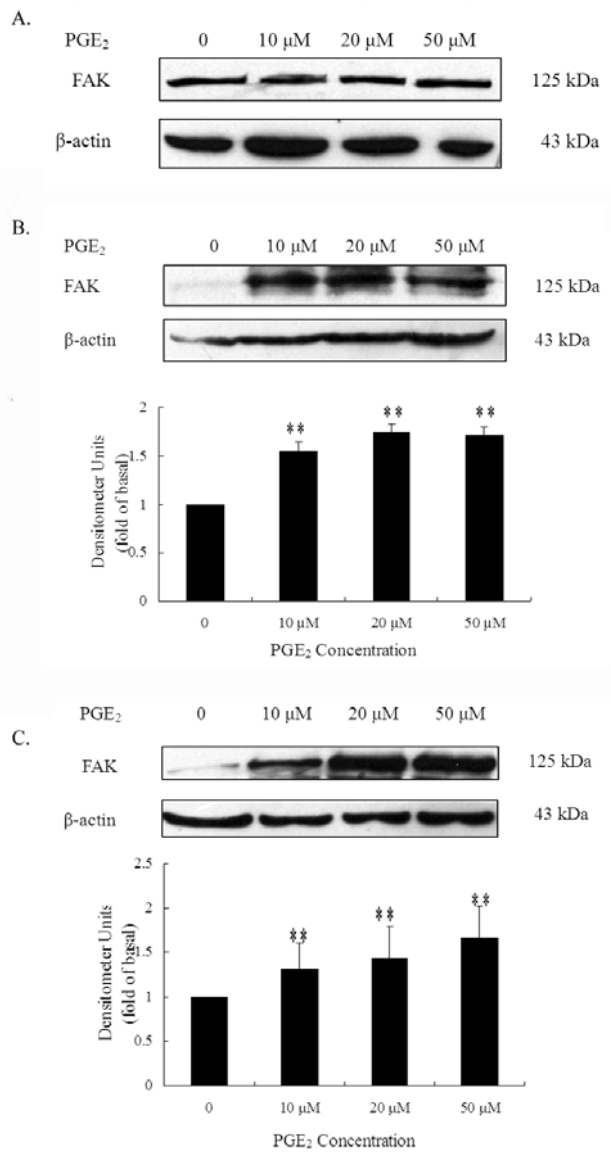

Figure 3. The effect of PGE_2 treatment on the FAK expression in HCC cells. HUH-7 cells were cultured, and treated with different concentrations of PGE_2. Levels of FAK and ß-actin were analyzed by Western blotting, and normalized to the amount of ß-actin by densitometric quantitation. The effects of PGE_2 treatment on the FAK expression for 8 (A), 12 (B) and 24 h (C). Results were the mean ± SD from three different experiments. **Significant difference at P<0.01 when compared to the cells without the treatment.

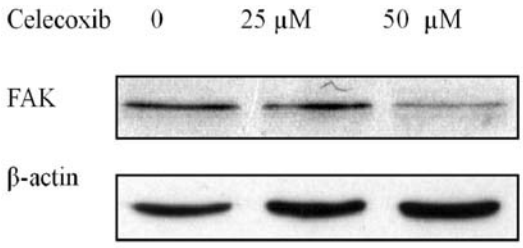

Figure 4. Celecoxib decreased the expression of FAK in HCC cells. HUH-7 cells were cultured and treated with different concentrations of celecoxib for 12 h. Levels of FAK and ß-actin were analyzed by Western blotting.

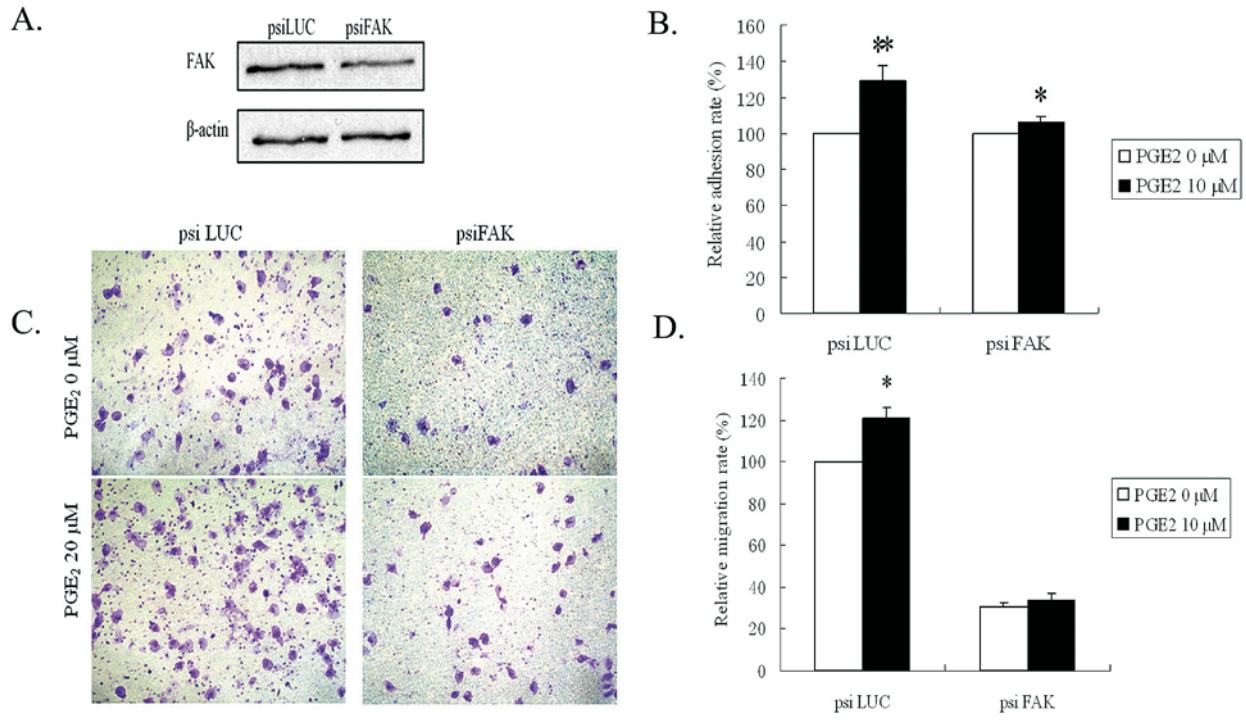

Figure 5. RNA interference targeting FAK suppressed PGE_2-mediated cell adhesion and migration. (A) Plasmid-encoded FAK small interfering RNA suppressed the expression of FAK in the cells. HUH-7 cells ($2x10^5$ cells) were seeded in 6-well plate, and cultured for 24 h before transfection. Cells were then cultured by serum-free DMEM. PsiFAK plasmid (4 μg) and 10 μl lipofectamine 2000 were mixed, and added to the cells. The constructed plasmid targeting luciferase (psiLUC) was used as a negative control. After the incubation for 4 h at 37˚C, cells were cultured in DMEM containing 10% serum for 48 h. Depletion of FAK was confirmed by Western blotting. These experiments were performed 3 times with similar results. (B) RNA interference targeting FAK inhibited PGE_2-mediated adhesion. After the transfection for 48 h, HUH-7 cells ($5x10^4$) were added to 96-well plates in 96-well plates in serum-free DMEM. After incubation for 3 h at 37˚C with PGE_2, the attached cells were stained with WST, and quantified by the absorbance at 450 nm. Results are mean ± SD from three different experiments. *Significant difference at P<0.05 when compared to the cells without treatment. **Significant difference at P<0.01 when compared to the cells without treatment. (C) RNA interference targeting FAK inhibited PGE_2-mediated migration. After the transfection for 48 h, HUH-7 cells ($5x10^4$ cells) were added to 12-well transwell in the upper chamber with or without PGE_2 in serum-free DMEM, and allowed to migrate for 12 h. HUH-7 cells on the lower surface were fixed by ethanol, and then stained by 0.1% crystal violet. Then the cells migrated to lower surfaces were taken pictures under the microscope (x100). (D) The migrated cells were solubilized with 10% acetic acid 300 μl and quantified by the absorbance at 570 nm. Results were mean ± SD from three different experiments. *Significant difference at P<0.05 when compared to the cells without treatment.

serum-free DMEM. PsiFAK (4 μg) and 10 μl lipofectamine 2000 were mixed, and added to the cells. The constructed plasmid targeting luciferase (psiLUC) was used as a negative control. After incubation for 4 h at 37˚C, cells were cultured in DMEM containing 10% serum for 48 h. Depletion of FAK was confirmed by Western blotting, and the cells were subsequently used for further experiments.

Statistical analysis. Data are presented as mean ± SD. P-values were calculated using the Student's t-test for unpaired sample led by MS Excel. The results were considered to be significantly different at P<0.05.

Results

PGE_2 increased cell adhesion, migration, and invasion in HCC. As shown in Fig. 1A, HUH-7 cell adhesion was enhanced with PGE_2 treatment, reaching ~1.6-fold after exposure to 20 μM PGE_2 for 3 h. The cell migration and invasion were studied using transwell assay. Cell migration was increased by 23% when the cells were treated with 20 μM PGE_2 for 12 h (Fig. 1B). Similarly the relative invasion rate analysis showed that PGE_2 treatment increased cell invasion by 1.3-fold when the cells were treated by 20 μM PGE_2 for 24 h (Fig. 1C). These results demonstrated that PGE_2 significantly promoted the cell adhesion, migration, and invasion.

PGE_2 induced FAK phosphorylation and expression. Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumor cells. To identify whether FAK was involved in PGE_2-induced cell adhesion and invasion, the FAK phosphorylation at Tyr397site was detected by Western blotting. When HUH-7 cells were exposed to PGE_2 for 30 min, FAK phosphorylation at Tyr397 was enhanced in a PGE_2-concentration-dependent manner (Fig. 2). In order to test whether PGE_2 could increase FAK protein expression, HUH-7 cells were treated by PGE_2 for different times. After 8h of PGE_2 treatment, FAK expression was not affected by PGE_2 (Fig. 3A). After 12 h of PGE_2 treatment, the levels of FAK expression were significantly increased in a PGE_2-concentration-dependent manner (Fig. 3B). PGE_2 at 10 μM and at 50 μM increased FAK expression by 1.5-fold and 1.7-fold,

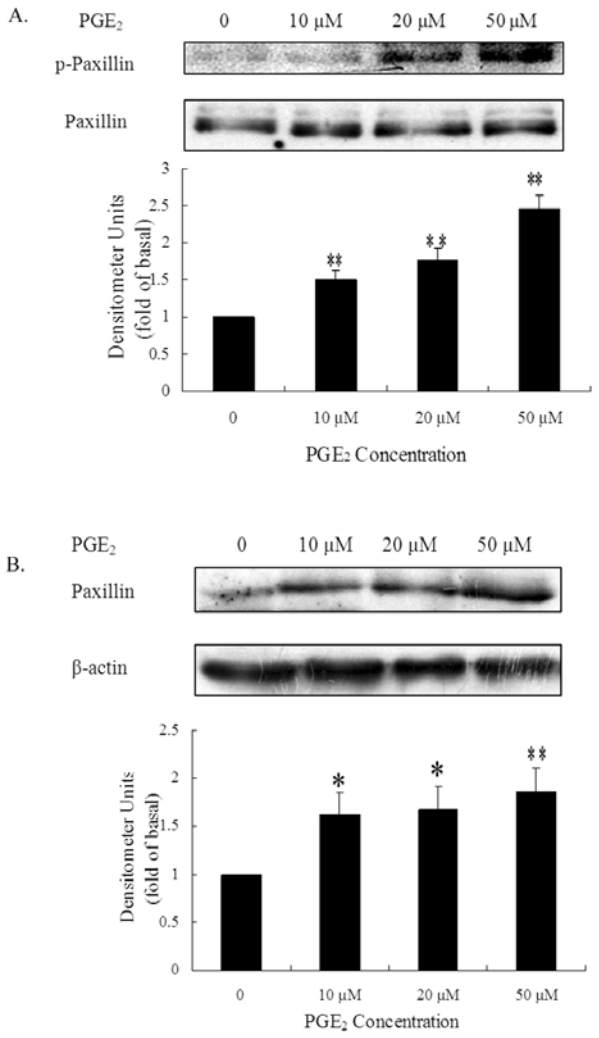

Figure 6. PGE_2 induced phosphorylation and expression of Paxillin in HCC cells. (A) PGE_2 induced paxillin phosphorylation at Tyr31. HUH-7 cells were cultured, and treated with different concentrations of PGE_2 for 30 min. Relative levels of phosphorylated Paxillin and total Paxillin expression were determined using anti-phospho-Paxillin and anti-Paxillin antibodies as described in the Materials and methods. (B) PGE_2 increased the expression of paxillin. HUH-7 cells were cultured, and treated with different concentrations of PGE_2 for 12 h. Densitometric quantitations of blots were shown as the mean ± SD from three different experiments. **Significant difference at P<0.01 when compared to the cells without treatment.

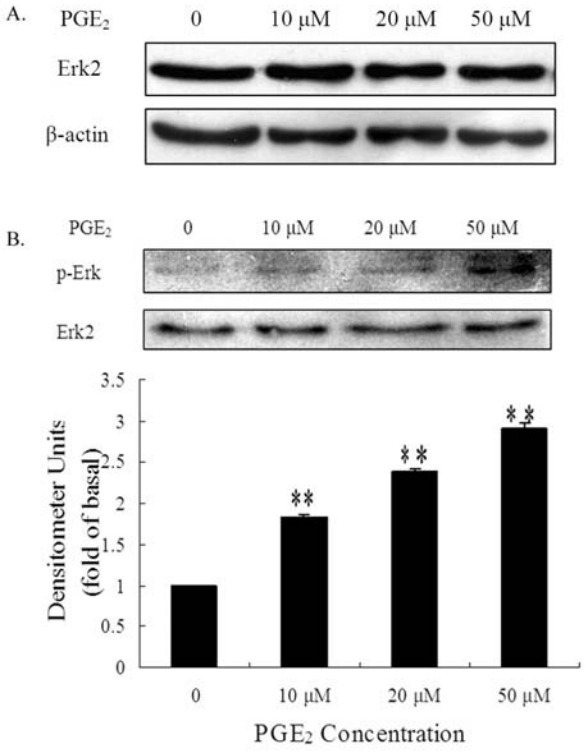

Figure 7. The effects of PGE_2 treatment on phosphorylation and expression of Erk in HCC cells. (A) The effect of PGE_2 treatment on the expression of Erk2. HUH-7 cells were cultured and treated with different concentrations of PGE_2 for 24 h. These experiments were performed 3 times with similar results. (B) The effect of PGE_2 treatment on phospho-Erk2 level. Erk2 phosphorylation is increased after stimulation with different concentrations of PGE_2 for 30 min. Relative levels of phosphorylated Erk and total Erk expression were determined using anti-phospho-Erk and anti-Erk2 antibodies as described in the Materials and methods. Densitometric quantitations of blots were shown as the mean ± SD from three different experiments. **Significant difference at P<0.01 when compared to the cells without treatment.

respectively. Similar result was obtained after 24 h of PGE_2 treatment.

Selective COX-2 inhibitor decreased FAK protein expression. Celecoxib is a specific selective COX-2 inhibitor. It can reduce PGE_2 production greatly in HCC (11). In this study, we used celecoxib to decrease the level of endogenous PGE_2, and then examined the potential effect of celecoxib on FAK expression in HCC cells. We found FAK protein expression was also decreased. As is shown in Fig. 4, 25 μM celecoxib caused ~30% reduction in FAK expression; 50 μM celecoxib almost completely blocked FAK expression.

RNA interference targeting FAK suppressed PGE_2-mediated cell adhesion and migration. As is shown in Fig. 5A, expression of FAK was suppressed by RNA interference when HUH-7 cells were transfected with psiFAK. After 48 h of transfection, HUH-7 cells ($5x10^4$) were added to 96-well plates and 12-well transwell units to detect their abilities of adhesion and migration. In the cell adhesion assay, 10 μM PGE_2 increased the cell adhesion by 30% in psiLUC-transfected cells, while only increased the adhesion by 6% in psiFAK-transfected cells (Fig. 5B).

In the cell migration assays, migrated cells were found much less in psiFAK-transfected cells than those in psiLUC-transfected cells without PGE_2 treatment. With 10 μM PGE_2 treatments, 20% more migrated cells were found in psiLUC-transfected group, but no effect was observed in psiFAK-transfected cells (Fig. 5C and D). These results illustrated that plasmid-encoded FAK small interfering RNA dramatically inhibited PGE_2-mediated cell adhesion and migration.

PGE_2 induced paxillin phosphorylation and expression. To identify whether the downstream signaling protein of FAK, paxillin, was involved in PGE_2-induced cell adhesion and invasion, the paxillin Tyr31 phosphorylation was detected by Western blotting. When HUH-7 cells were exposed to

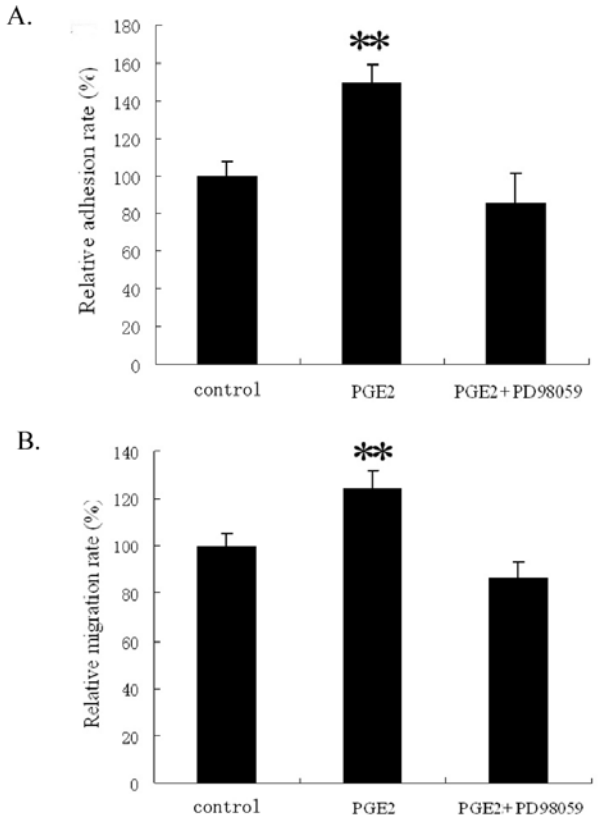

Figure 8. PD98059 blocked PGE_2-induced cell adhesion and migration in HUH-7 cells. (A) PD98059 suppressed PGE_2-induced cell adhesion. HUH-7 cells ($5x10^4$) were placed in 96-well plates in serum-free DMEM. PD98059 (25 μM) or PBS was added to each well. After incubation for 1 h at 37˚C, the cells were treated with 10 μM PGE_2. After treatment for 3 h at 37˚C, the attached cells were stained with WST, and quantified by the absorbance at 450 nm. (B) PD98059 suppressed PGE_2-induced cell migration. Cells ($5x10^4$) were placed in the upper chamber of 12-well transwells. PD98059 (25 μM) or PBS was added to each well. After incubation for 1 h at 37˚C, the cells were treated with 10μM PGE_2 and allowed to migrate for 12 h. HUH-7 cells on the lower surface were fixed by ethanol solution, and then stained by 0.1% crystal violet. The migrated cells were solubilized with 300 μl of 10% acetic acid, and quantified by the absorbance at 570 nm. Results were mean ± SD from three different experiments. **Significant difference at $P<0.01$ when compared to the cells without treatment.

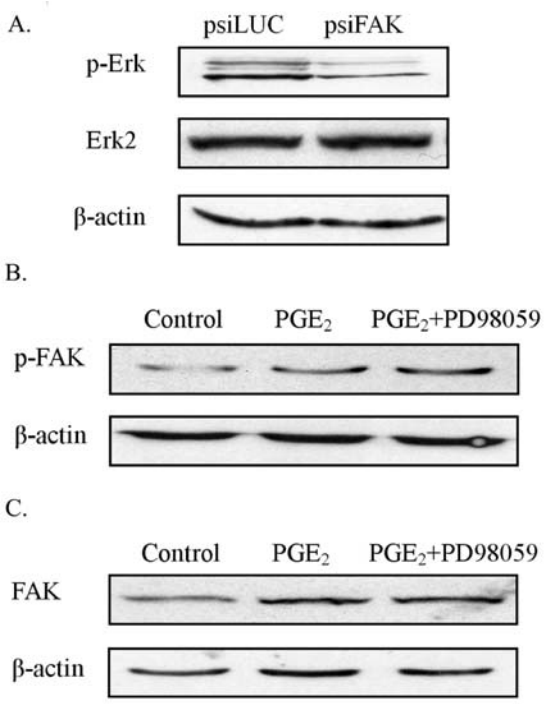

Figure 9. PD98059 had no effect on PGE_2-induced FAK activation and synthesis in HUH-7 cells. (A) RNA interference targeting FAK decreased Erk2 phosphorylation. HUH-7 cells ($2x10^5$ cells) were seeded in 6-well plate, and cultured for 24 h before the transfection. Cells were then transfeted in serum-free DMEM with PsiFAK plasmid (4 μg) and 10 μl lipofectamine 2000. The constructed plasmid targeting luciferase (psiLUC) was used as a negative control. After the incubation for 4 h at 37˚C, cells were cultured in DMEM containing 10% serum for 48 h. Suppression of Erk phosphorylation was confirmed by Western blotting. These experiments were performed 3 times with similar results. (B and C) The effects of PD98059 on PGE_2-induced FAK phosphorylation and expression. HUH-7 cells ($2x10^5$ cells) were seeded in 6-well plate, and cultured for 24 h before treatment. PD98059 (25 μM) or PBS was added to each well. After incubation for 1 h at 37˚C, the cells were treated with 10 μM PGE_2 for 30 min and 24 h, respectively. Phosphorylation and expression of FAK were confirmed by Western blotting. These experiments were performed 3 times with similar results.

PGE_2 for 30 min, paxillin Tyr31 phosphorylation was enhanced in a PGE_2-concentration-dependent manner (Fig. 6). As shown in Fig. 6A, 10 μM and 50 μM PGE_2 treatments increased paxillin phosphorylation at Tyr31 by 34 and 250%, respectively.

In order to clarify if PGE_2 could stimulate paxillin protein expression, HUH-7 cells were treated by PGE_2 for different times. The levels of paxillin expression were increased in a PGE_2-concentration-dependent manner. PGE_2 at 10 μM and at 50 μM increased paxillin expression by 1.6-fold and 1.8-fold, respectively (Fig. 6).

Erk phosphorylation was involved in PGE_2-mediated cell adhesion and migration. It was reported that FAK required the activation of extracellular signal-regulated kinase (Erk) to regulate cell growth in tumor cells (17). Our results showed that when the cells were exposed to PGE_2, the expression of Erk was not changed (Fig. 7A), but Erk2 phosphorylation was induced after treatment with PGE_2 for 30 min (Fig. 7B). When HUH-7 cells were treated with 10 μM PGE_2, the phospho-Erk2 level was increased by 30%; and with 50 μM PGE_2, it reached maximum response by >2-fold higher than the control (Fig. 7B). No significant change was seen in the phospho-Erk1 level after PGE_2 treatment.

PD98059 was specific inhibitor of MAPK p44/42 pathway. The abilities of adhesion and migration were analyzed following pretreatment of 25 μM PD98059. As shown in Fig. 8, treatment with PD98059 could effectively block PGE_2-induced cell adhesion and migration.

In order to clarify whether Erk2 was downstream of PGE_2-induced FAK/paxillin pathway, we detected the effect of PD98059 on FAK phosphorylation and expression after PGE_2 treatment. Fig. 9A shows that when HUH-7 cells were transfected with psiFAK, the phospho-Erk2 level was greatly decreased. However, PD98059 had no effect on PGE_2-induced upregulation of FAK phosphorylation and expression in HUH-7 cells (Fig. 9B and C).

Discussion

Spread and metastasis are the most deadly aspects of cancers. PGE_2 have been proved to induce the cancer cell proliferation, and migration in many types of cancer cells (12-15). However, the effects of PGE_2 on migration and invasion and its mechanism have not been clarified yet in HCC. Our studies showed that PGE_2 greatly induced cell adhesion, migration, and invasion, and these effects were associated with the phosphorylation and synthesis of focal adhesion kinase and its downstream protein, paxillin.

Focal adhesion kinase is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of cell proliferation and migration. FAK appears to be essential for the regulation of cell adhesive and migratory properties (20-23). It associates with integrin receptors, and integrates other molecules by forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton (21,22). The ability of FAK to combine integrin and growth factor signals leads to synergistic promotion of cell migration and proliferation (17). It has been reported that PGs produced were implicated in cell migration and secretion of MMP mainly through integrin αVß3 (15). In our studies, we proved evidence that FAK also played a key role in PGE_2-mediated cell adhesion, migration and invasion in hepatocellular carcinoma cell line.

For integrin-stimulated cell migration, the FAK phosphorylation at Tyr397 and its protein expression were required (17,24). In our studies, exogenous PGE_2 greatly increased the FAK phosphorylation at Tyr397 as well as its protein expression in the PGE_2-concentration-dependent manner. At the same time, selective COX-2 inhibitor celecoxib decreased the expression of FAK protein. It implied that FAK phosphorylation and expression were regulated by the level of PGE_2 in HCC cells. Further, RNA interference targeting FAK greatly suppressed PGE_2-mediated adhesion and migration. These results suggested that FAK may play an important role in PGE_2-mediated cell adhesion, migration and invasion.

FAK directly phosphorylates several proteins, such as the N-Wiskott Aldrich syndrome protein (N-WASP), talin, and paxillin (25). A splice variant of FAK, the FAK related non-kinase (FRNK) overexpression reduced the phosphorylation of FAK, and then inhibited the phosphorylation of paxillin (17,18). Paxillin was initially characterized as a 68-kDa focal adhesion protein. A principal function for paxillin is to integrate and disseminate signals from integrins to affect efficient cellular migration. Paxillin is an important mediator of signal cross-talk between MAPK families and small GTPases families through its phosphorylation and multipotent associations (26). Paxillin and talin also act together with FAK to link integrin receptors to the cytoskeleton (17). Paxillin is phosphorylated on multiple tyrosine (PY), serine (PS) and threonine (PT) residues in response to cell adhesion (26). Phosphorylation of serine and threonine residues is more likely to influence paxillin conformation and thereby allosterically affect its ability to interact with specific binding partners. In contrast, tyrosine phosphorylation generates docking sites for SH2 domain to facilitate downstream signaling. The most extensively characterized SH2 domains is pY31 and pY118 (27,28). In our studies, paxillin phosphorylation was increased in Tyr31 site, and these responses were in a PGE_2-concentration-dependent manner. It implicated that there may be other downstream proteins being activated by paxillin in PGE_2-mediated cell adhesion and migration.

In our present studies, the short-term treatment of PGE_2 greatly stimulated the phosphorylation of paxillin; and the long-term treatment increased the synthesis of paxillin in the PGE_2-concentration-dependent manner. At the same time, the levels of phospho-Erk2 were increased by PGE_2 treatments. Furthermore, pretreatment with PD98059, the specific MEK inhibitor, significantly blocked the effects of PGE_2 on cell adhesion and migration. This result suggested that activation of Erk2 was also involved in the PGE_2-mediated cell adhesion and migration.

It was reported that extracellular signal-regulated kinase is also a kind of downstream protein of FAK (17). FAK required the activation of Erk to regulate cell growth in tumor cells (29,30). FAK-Src signaling regulates adhesion in fibroblast cells through paxillin and Erk (31). The formation of FAK/paxillin/Erk complex was also showed to be implicated in proepithelin-mediated migration and invasion in bladder cancer cells (32). In our studies, we also found that phosphorylation of Erk2 was reduced when expression of FAK was suppressed by RNA interference. It suggested that FAK regulated phosphorylation of Erk in HCC. However, PD98059, the specific MEK inhibitor, could not influence PGE_2-induced upregulation of FAK phosphorylation and expression in HUH-7 cells. These results suggested that Erk2 was the downstream protein of PGE_2-activated FAK/paxillin pathway.

In summary, our results demonstrated that PGE_2 promoted cell adhesion, migration, and invasion in hepatocellular carcinoma cells, and that FAK, paxillin and Erk2 were involved in these processes. The FAK/paxillin/Erk2 signal pathway may play a key role in PGE_2-mediated cell migration and invasion. To our knowledge, this is the first report on the association of PGE_2 and FAK and its downstream pathway in tumor cells. These findings suggest a potential new target for therapeutic options in human HCC.

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