Inhibition of focal adhesion kinase induces apoptosis in bladder cancer cells via Src and the phosphatidylinositol 3-kinase/Akt pathway

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Abstract. Focal adhesion kinase (FAK) is a 125-kDa, cytosolic, non-receptor, protein tyrosine kinase localized at focal adhesions that can be activated by multiple inputs and in different manners. FAK is implicated in signaling pathways regulating cell movement, invasion, survival, gene expression and cancer stem cell self-renewal. The aim of the present study was to investigate whether FAK plays a role in the apoptosis of bladder cancer cells. The study employed *in situ* deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and Annexin V labeling flow cytometry. It was found that both the knockdown of FAK and the suppression of FAK phosphorylation were able to induce apoptosis in bladder cancer cells. Caspase-3 was activated during the apoptosis induced by the suppression of FAK phosphorylation. Src was involved in FAK-regulated apoptosis in bladder cancer cells, while the suppression of Src phosphorylation was able to inhibit FAK tyrosine phosphorylation and induce apoptosis. Furthermore, phosphatidylinositol 3-kinase (PI3K)/Akt signaling was inhibited via the suppression of FAK tyrosine phosphorylation. Conversely, the expression of neither the general nor the tyrosine-phosphorylated FAK was regulated by inhibiting PI3K/Akt, which suggested that PI3K/Akt acted downstream of FAK to regulate apoptosis in bladder cancer cells. These findings indicate the presence of a mechanism of apoptosis involving FAK-mediated oncogenic signaling. FAK may function as an important regulator of extracellular signaling-mediated apoptosis in bladder cancer and be used as a novel therapeutic target in the treatment of the condition.

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Introduction

Bladder cancer is the second most common genitourinary cancer and the eleventh most common malignancy worldwide. It has been estimated that ~429,800 new cases of bladder cancer and 165,100 bladder cancer-related mortalities occurred in 2012 worldwide, accounting for 3% of the total new cancer cases (1). Furthermore, bladder cancer is now the most frequent cancer of the urinary tract and the seventh most frequent malignancy in men worldwide. An increasing trend in the incidence and mortality rates of bladder cancer has been noted in the past 30 years (2). Among all newly diagnosed cases, ~75% of patients exhibit a non-muscle-invasive tumor without invasion into bladder detrusor; however, ~25% of patients present with muscle-invasive bladder tumors, which means that the bladder detrusor has been invaded by the cancer (3). Furthermore, between 50 and 70% of the cases of non-muscle-invasive tumors will recur following transurethral resection, despite intravesical chemotherapy or Bacillus Calmette-Guérin immunotherapy, and 10-20% will progress to muscle-invasive bladder tumors in 5 years (4). Notably, lymph node metastases are confirmed by pathological examination in ~25% of patients with muscle-invasive cancer who have undergone radical cystectomy, while it is estimated that one-third of patients with muscle-invasive cancer have undetected metastases at first diagnosis (5). There is, therefore, an active interest in the study of the molecular mechanism of bladder cancer.

Focal adhesion kinase (FAK) is a 125-kDa tyrosine kinase found in focal adhesions. It is a member of a growing family that includes several structurally distinct protein tyrosine kinases, such as related adhesion focal tyrosine kinase and calcium-dependent protein tyrosine kinase (6). FAK can be activated by specific extracellular stimuli, such as integrins and certain growth factors (7). FAK is encoded by the protein tyrosine kinase 2 (PTK2) gene, which is located at human chromosome region 8q24.3. It has been found that the region is commonly amplified in several types of cancer, such as serous ovarian (8)

and gastric (9) cancer. Abnormally increased FAK expression has additionally been found in several types of cancer (10), such as cervical (11), ovarian (12), breast (13) and lung (14) cancer. FAK plays an important role in signal transduction in the tumor microenvironment (15), as it is a multifunctional scaffolding molecule that links transmembrane input signals from growth factor receptors and integrins to intracellular effectors, such as c-Jun N-terminal kinase and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) (16). FAK proteins are known to regulate cell survival and apoptosis via several pathways, such as PI3K/Akt (17).

An increased FAK mRNA level is found in bladder cancer (10); however, no study to date has been performed to determine whether FAK is associated with the survival and apoptosis of bladder cancer cells. The aim of the present study, therefore, was to explore the potential role of FAK in the apoptosis of bladder cancer cells.

Materials and methods

Cell culture and reagents. The T24 human bladder cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum at 37˚C in a humidified 5% CO_2/95% air atmosphere. The FAK inhibitor PF-573228 (PF-228), the Src inhibitor PP2 and the PI3K inhibitor LY294002 were obtained from Sigma-Aldrich (St. Louis, MO, USA). TGFβ was obtained from Peprotech, Inc. (Rocky Hill, NJ, USA).

Preparation and transfection of small interfering RNAs (siRNAs). siRNAs against FAK [sense, 5'-UAAUACUCGCUC CAUUGCACC(dT)(dT)-3' and antisense, 5'-GGUGCAAUG GAGCGAGUAUUA(dT)(dT)-3'] were designed as described previously (18). The siRNA duplexes were chemically synthesized by GeneChem Co., Ltd. (Shanghai, China). Transfections were performed in six-well plates (Corning Costar, Cambridge, MA, USA) with Lipofectamine™ 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR). The treated cells were collected and the total RNA was prepared using TRIzol® reagent (Invitrogen Life Technologies). Subsequently, reverse transcription was performed with 2 μg total RNA using a one-step RT-PCR system (Invitrogen Life Technologies). qPCR was conducted in an Applied Biosystems 7300 Real-time PCR Instrument (Applied Biosystems Life Technologies, Foster City, CA, USA). The PCR cycling conditions were as follows: 95˚C for 3 min, followed by cycles at 95˚C for 10 sec and 60˚C for 20 sec, then 72˚C for 15 sec. The PCR products were detected using SYBR® Green dye (Applied Biosystems Life Technologies) according to the manufacturer's instructions. Specific oligonucleotide primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Relative quantification of the mRNA levels of target genes was performed using the $2^{-\Delta\Delta CT}$ method, as described previously (19).

Western blot analysis. Western blot analysis was performed as described previously (20). Total protein (50 μg) from each sample was loaded for SDS-PAGE. The membrane was exposed on an X-ray film (Eastman Kodak Co., Rochester, NY, USA) using enhanced chemiluminescence western blot detection reagents (Pierce Biotechnology, Inc., Rockford, IL, USA). Cumulative gray levels of all bands were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for further relative quantitative analysis. Primary antibodies that were specific against following proteins were used: Rabbit polyclonal FAK (#3285; 1:1,000), rabbit polyclonal phosphorylated (p)FAK (#3283; 1:1,000), rabbit mAb Src (#2109; 1:1,000), rabbit polyclonal pSrc (#2101; 1:1,000), mouse mAb Akt (#2966; 1:500), mouse mAb pAkt (#4051; 1:1,000), mouse mAb caspase-3 (#9668; 1:1,000) and cleaved caspase-3 (#9579; 1:1,000) (all Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit polyclonal β-actin (sc-1616-R; 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was incubated with primary antibodies at 4˚C overnight. Horseradish peroxidase-labeled secondary antibodies were obtained from Santa Cruz Biotechnology, Inc.

In situ deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. *In situ* TUNEL assay was employed to detect the apoptotic cells. T24 cells were grown on slides and fixed with 4% buffered formaldehyde. TUNEL assay was performed using the *in situ* Cell Death Detection kit (Boehringer Mannheim GmbH, Mannheim, Germany) as described previously (18). The slides were counterstained with hematoxylin. The apoptotic cells were stained brown under the microscope.

Annexin V labeling. To measure the numbers and the ratio of apoptotic cells, the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was employed as described previously (18). The treated cells were stained with FITC, Annexin V and propidium iodide (PI), and the stained cells were analyzed using a FACSort™ flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and evaluated with the CellQuest™ software system (BD Biosciences).

Cell viability assay. Cell viability was determined using the MTT assay as described previously (18). Cells were cultured in 96-well plates at a density of $2x10^4$ cells/well. The cell viability was measured using the MTT assay. Cells were incubated with 10 μl 0.5 mg/ml MTT at 37˚C for 4 h. The formazan crystals were dissolved using 200 μl dimethylsulfoxide and quantified by measuring absorbance at 570 nm.

Statistical analysis. All experiments were repeated at least three times, and the data were analyzed using the SPSS 12.0 statistical software package (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and the Student's *t*-test were employed to compare the data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of FAK induces apoptosis in T24 bladder cancer cells. To identify whether FAK affected the survival or apoptosis of T24 bladder cancer cells, FAK expression in

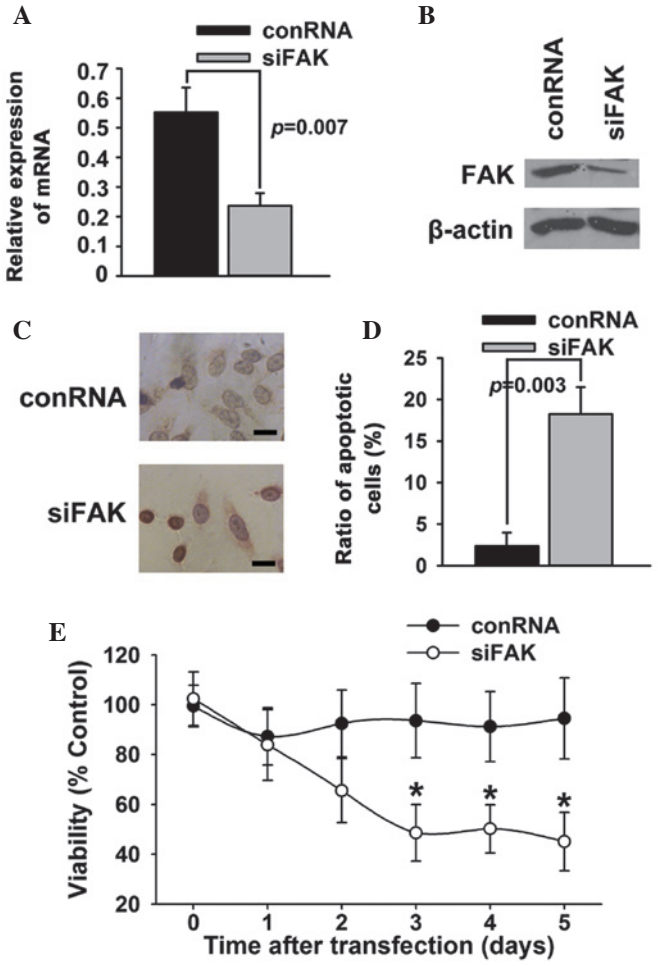

Figure 1. Knockdown of FAK induces apoptosis in T24 bladder cancer cells. T24 bladder cancer cells were transfected with small interfering RNA against FAK (siFAK) or control (conRNA). (A and B) The expression of FAK was examined using western blotting. (C and D) Cell apoptosis was examined using (C) deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay and (D) Annexin V/propidium iodide. (E) Cell survival was examined using an MTT assay. Scale bar, 200 μm. *P<0.05. FAK, focal adhesion kinase.

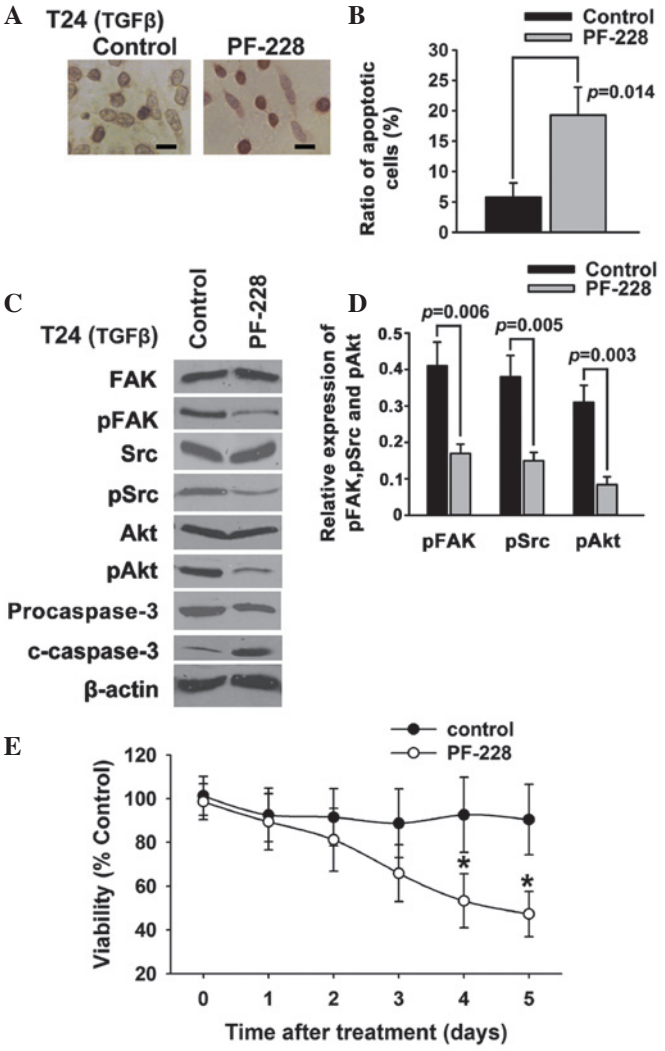

Figure 2. Suppression of FAK phosphorylation induces apoptosis in T24 bladder cancer cells. T24 bladder cancer cells were treated with PF-228 and 5 ng/ml TGFβ. (A and B) Cell apoptosis was examined using (A) deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay and (B) Annexin V/propidium iodide. (C and D) The expression of FAK, pFAK, Src, pSrc, Akt, pAkt, caspase-3 and c-caspase-3 was examined using western blotting. (E) Cell survival was examined using an MTT assay. Scale bar, 200 μm. *P<0.05. FAK, focal adhesion kinase; pFAK, phosphorylated FAK; c-caspase-3, cleaved caspase-3; TGFβ, transforming growth factor-β.

T24 bladder cancer cells was knocked down using a specific siRNA duplex targeting FAK. As shown in Fig. 1A and B, the siRNA duplex caused a marked decrease of FAK mRNA and protein expression in the T24 bladder cancer cells. The results of TUNEL (Fig. 1C) and Annexin V/PI (Fig. 1D) assays showed that siRNA against FAK significantly increased the apoptosis of T24 bladder cancer cells. The results of the MTT assays (Fig. 1E) showed that inhibition of FAK expression by siRNA decreased the viability of the T24 cells compared with control RNA.

Suppression of FAK phosphorylation induces apoptosis in T24 bladder cancer cells. To further investigate whether the inhibition of FAK tyrosine phosphorylation could promote the apoptosis of T24 bladder cancer cells, PF-228, a selective inhibitor of FAK, was employed to inhibit the transforming growth factor-β (TGFβ)-induced tyrosine phosphorylation of FAK. The results of the TUNEL (Fig. 2A) and Annexin V/PI (Fig. 2B) assays showed that PF-228 was able to induce the apoptosis of T24 bladder cancer cells. The western blotting results indicated that PF-228 significantly reduced the TGFβ-induced phosphorylation of FAK and Src, suppressed the phosphorylation of Akt, an accepted signal of cell survival/apoptosis, and activated caspase-3, an important apoptosis-related protein (Fig. 2C and D). The results of the MTT assays (Fig. 2E) showed that inhibition of FAK by PF-228 decreased the viability of the T24 cells compared with the control.

Src is an important mediator of FAK-regulated apoptosis in T24 bladder cancer cells. FAK is a substrate for the oncogene protein tyrosine kinase Src (21). To further whether the inhibition of Src phosphorylation could also promote the apoptosis of T24 bladder cancer cells, PP2, a selective inhibitor of Src, was employed to inhibit the TGFβ-induced phosphorylation of Src. The results of the TUNEL (Fig. 3A) and Annexin V/PI (Fig. 3B) assays showed that PP2, similarly to the FAK inhib-

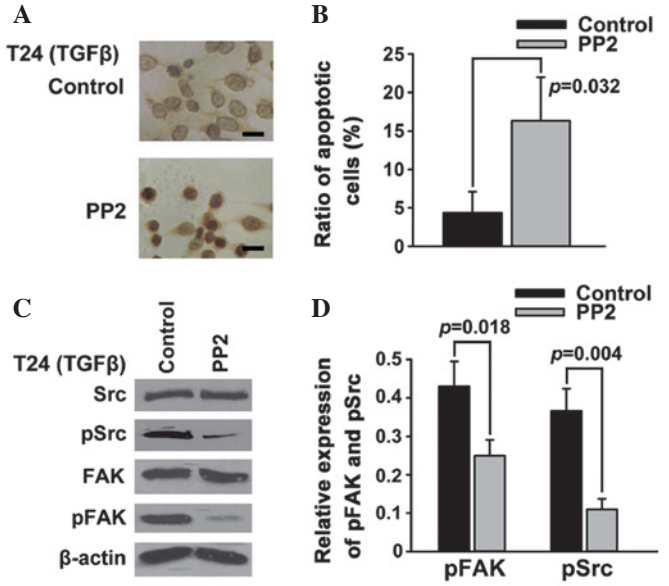

Figure 3. Src is an important mediator of FAK-regulated apoptosis in T24 bladder cancer cells. T24 bladder cancer cells were treated with PP2. (A and B) Cell apoptosis was examined using (A) deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay and (B) Annexin V/propidium iodide. (C and D) The expression of FAK, pFAK, Src and pSrc was examined using western blotting. Scale bar, 200 μm. FAK, focal adhesion kinase; pFAK, phosphorylated FAK; TGFβ, transforming growth factor-β.

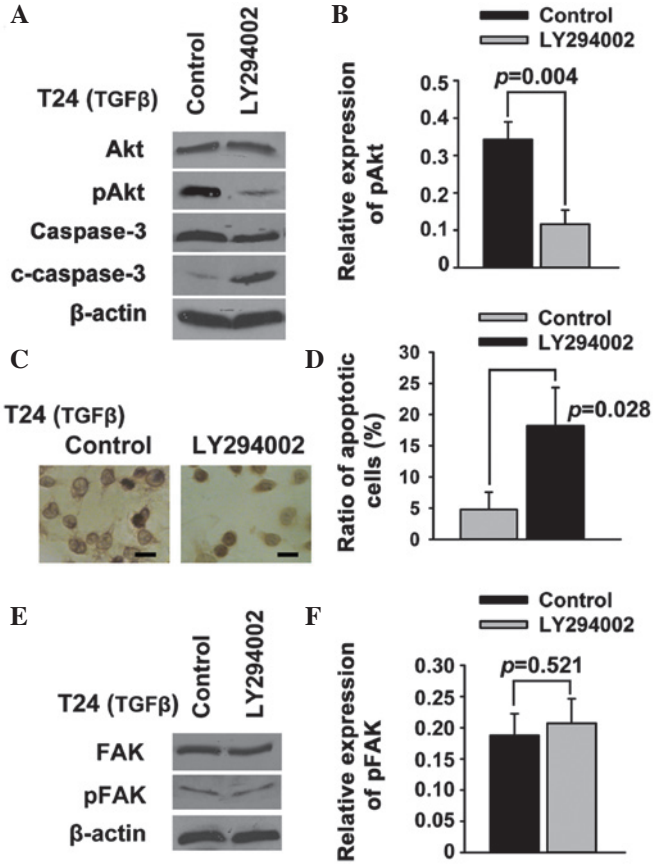

Figure 4. Phosphatidylinositol 3-kinase/Akt acts downstream of FAK signaling to regulate apoptosis in T24 bladder cancer cells. T24 bladder cancer cells were treated with LY294002. (A and B) The expression of Akt, pAkt, caspase-3 and c-caspase-3 was examined using western blotting. (C and D) Cell apoptosis was examined using (C) deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay and (D) Annexin V/propidium iodide. (E and F) The expression of FAK and pFAK was examined using western blotting. Scale bar, 200 μm. FAK, focal adhesion kinase; pFAK, phosphorylated FAK; c-caspase-3, cleaved caspase-3; TGFβ, transforming growth factor-β.

itor PF-228, was able to induce the apoptosis of T24 bladder cancer cells. The western blotting results demonstrated that PP2 significantly reduced the TGFβ-induced phosphorylation of not only Src but also FAK (Fig. 3C and D).

PI3K/Akt signaling acts downstream of FAK to regulate apoptosis in T24 bladder cancer cells. PI3K/Akt is a potent pathway of survival and apoptosis (22), and FAK is believed to be an upstream signal protein of the PI3K/Akt pathway (23); therefore, it was investigated whether PI3K/Akt acted downstream of FAK to regulate the apoptosis of T24 bladder cancer cells. Western blot analysis showed that Akt and FAK phosphorylation was suppressed by PF-228 (Fig. 2C and D). In addition, LY294002 significantly downregulated pAkt in the T24 cells (Fig. 4A and B). The results of the TUNEL (Fig. 4C) and Annexin V/PI (Fig. 4D) assays showed that LY294002 was able to induce the apoptosis of T24 cells. Conversely, the expression of general and tyrosine-phosphorylated FAK was not regulated by inhibiting PI3K/Akt in T24 cells (Fig. 4E and F).

Discussion

FAK was first described by Linder and Burr in 1988 as a 120-kDa protein that was one of a large number of tyrosine phosphoproteins in Rous sarcoma virus-transformed chicken embryo fibroblasts (24). Reynolds *et al* (25) reported the finding of a 120-kDa protein whose phosphorylation was greatly enhanced in cells expressing activated, oncogenic Src in 1989, and the same research group generated monoclonal antibodies against the 120-kDa protein in 1990 (26). Furthermore, a 120-kDa protein was described to tyrosine-phosphorylate in fibronectin-stimulated cells by Guan *et al* in 1991 (27). The protein was located in focal contacts, where it codistributed with β1 integrins. Phosphorylation of the protein was correlated with subsequent cell spreading. It was suggested that the interaction of β1 integrins with extracellular ligands, such as fibronectin, turned on the phosphorylation of the 120-kDa protein, which may have been involved in the responses of cells to attachment. Schaller *et al* (28) identified a 125-kDa phosphotyrosine-containing protein as a tyrosine phosphatase substrate of v-Src in chicken embryo cells in 1992. In the study by Schaller *et al*, cDNA of the protein was isolated, and the predicted structure of the new protein, which was the prototype for an additional family of protein-tyrosine kinases, was found. Since the protein was localized to focal adhesions, they named the new protein focal adhesion kinase. In the same year, Hanks *et al* (29) found that the activation of FAK via tyrosine phosphorylation was an important early step in intracellular signal transduction pathways in response to extracellular stimuli with the extracellular matrix. In 1994, Schaller *et al* (30) suggested that Tyr-397 was a major site of FAK autophosphorylation and that FAK was physically associated with Src via their SH2 domains. More recent research has shown that FAK links extracellular stimuli, such as integrins

and growth factors, to intracellular signaling and regulates cell movement, migration, invasion, survival and cancer stem cell self-renewal (10,31). The aim of the present study was to determine if FAK regulated the survival and apoptosis of bladder cancer cells.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms and is characterized by morphological changes of the cells, include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (32). The activation of FAK by the interaction of extracellular matrix signals and integrins has been shown to be accompanied by suppressed apoptosis in diverse cell types (33). Notably, FAK was found to be tyrosine-phosphorylated by oxidative stress prior to the occurrence of apoptosis (34). Sonoda *et al* (34) found that FAK retained tyrosine phosphorylation at least up to 5 h and gradually lost tyrosine phosphorylation after 8 h, concomitant with apoptosis. While FAK was inhibited by inhibitor of protein tyrosine kinases or antisense oligonucleotide against FAK, apoptosis was accelerated. Sonoda *et al* suggested that tyrosine phosphorylation of FAK played a suppressive role in cell apoptosis. Since it has been shown that specific siRNAs targeting mRNA of the PTK2 gene are effective in inhibiting the expression of FAK (35-39), RNA interference was employed in the present study as a potent tool to explore the role of FAK in the survival and apoptosis of bladder cancer cells. The results showed that apoptosis was induced in T24 bladder cancer cells when FAK was suppressed by siRNA targeting FAK. Similar results were observed following the administration of PF-228, an exclusive inhibitor of FAK tyrosine phosphorylation. These results suggest that not only the general expression but also the tyrosine phosphorylation level of FAK is associated with the apoptosis of bladder cancer cells.

FAK is an important mediator of TGFβ signaling (40). TGFβ predominantly regulates FAK via tyrosine phosphorylation of FAK (41). FAK was first described as a 120-kDa protein that was tyrosine-phosphorylated following induction by Src (24). FAK is autophosphorylated at Tyr-397 due to input signals from integrins and growth factor receptors, to which Src proteins subsequently bind. Afterwards, FAK-Src complexes are formed, and FAK is phosphorylated at Tyr-576 and Tyr-577. The complexes play an important role in mediating the extracellular signal to downstream molecules such as extracellular signal-regulated kinases and paxillin (42,43); therefore, Src is one of the most important regulatory proteins in FAK-related signals. PP2 is a selective inhibitor of Src family members and is able to block the Tyr-416 phosphorylation of Src (44). In the present study, therefore, the effect of Src tyrosine dephosphorylation on FAK and the apoptosis of bladder cancer cells was explored using PP2. The results showed that PP2 was able to induce the apoptosis of T24 cells, while tyrosine phosphorylation of not only Src but also FAK was inhibited in TGFβ-stimulated bladder cancer cells.

Notably, Wen *et al* (45) found that FAK is cleaved into two different fragments in early apoptosis, which is mediated by caspase-7 and caspase-3 (45). They suggested that the disruption of FAK may contribute to the morphological changes of cells in apoptosis. Levkau *et al* (46) found that cleavage of FAK affected its association with signaling and other cytoskeletal components of the focal adhesion complex, such as paxillin. They suggested that the caspase-mediated cleavage of FAK disturbed survival signals from the extracellular matrix and propagated the cell death program. Van de Water *et al* (47) found that the inhibition of caspase activity blocked FAK cleavage and apoptosis, but not FAK dephosphorylation. They suggested that caspases are required for FAK cleavage, but not for FAK dephosphorylation, during apoptosis. Sonoda *et al* (23) demonstrated that the tyrosine phosphorylation of FAK, the association of FAK with PI3K and the serine phosphorylation of Akt occurred during oxidative stress-induced apoptosis. They suggested that FAK was an upstream signal protein of the PI3K/Akt pathway during oxidative stress-induced apoptosis. In another study, Sonoda *et al* (48) found that the PI3K/Akt survival pathway was activated, and the activation of procaspase-3 to caspase-3 was inhibited, in FAK-transfected cells, which had resistance to apoptotic stimuli. They suggested that FAK activated the PI3K/Akt survival pathway, as well as its downstream signals, and finally inhibited apoptosis by blocking the caspase-3 cascade. The role of the PI3K/Akt pathway and caspase-3 during the apoptosis of bladder cancer cells induced by inhibiting FAK was therefore explored in the present study. The results showed that the phosphorylation of Akt was suppressed and the activation of procaspase-3 to caspase-3 was induced during apoptosis, while FAK was dephosphorylated by a tyrosine phosphorylation inhibitor. LY294002, a common inhibitor of PI3K, was additionally utilized to investigate the effect of inhibiting the PI3K/Akt pathway on the general expression and tyrosine phosphorylation level of FAK. The results showed that inhibiting the PI3K/Akt pathway was able to induce the apoptosis of T24 cells, but did not regulate either the general expression or the tyrosine phosphorylation of FAK. These results suggested that PI3K/Akt acted downstream of FAK signaling to regulate apoptosis in bladder cancer cells.

In this study, the regulatory role of FAK signaling on the apoptosis and survival of bladder cancer cells was demonstrated. Both the knockdown of FAK and the suppression of FAK phosphorylation were able to induce apoptosis in bladder cancer cells. Caspase-3 was activated during the apoptosis induced by the suppression of FAK phosphorylation. Src was involved in FAK-regulated apoptosis in bladder cancer cells, while the suppression of Src phosphorylation was able to inhibit FAK tyrosine phosphorylation and induce apoptosis. Furthermore, PI3K/Akt signaling was inhibited via the suppression of FAK tyrosine phosphorylation. Conversely, neither the expression of general nor tyrosine-phosphorylated FAK was regulated by inhibiting PI3K/Akt. These results suggested that PI3K/Akt acted downstream of FAK signaling to regulate apoptosis in bladder cancer cells. Collectively, the data indicate that FAK is an important regulator of apoptosis and survival signaling in bladder cancer cells.

Acknowledgements

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