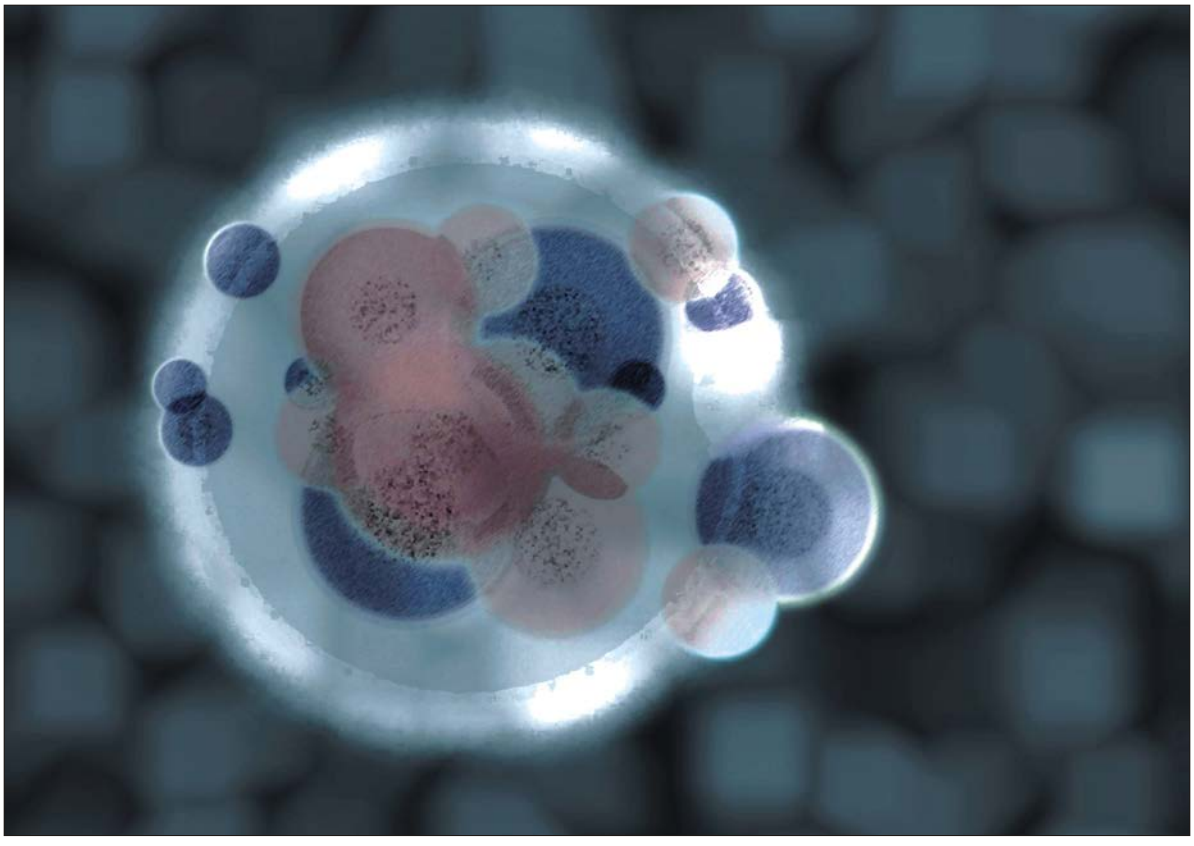

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Differential Phosphorylation and Expression Events of Focal Adhesion Kinase under Anchorage-dependent and Anchorage-independent Conditions

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Abstract: Focal Adhesion Kinase (FAK) is a nonreceptor protein tyrosine kinase which plays an important role in anchorage-dependent growth and survival. However, little is known about FAK involvement in the progression of leukemic diseases as an example of anchorage-independent malignant diseases. In this study, we commence to examine FAK protein expression and phosphorylation in Hepatocellular carcinoma and leukemic cell lines, Hep G2 and U-937 respectively, using Western blotting analysis. In addition, Hep G2 was subjected to immunofluorescence analysis to study the subcellular localization of FAK and phosphorylated FAK proteins in the cell line. This study showed that the total FAK protein was found to be expressed in both cell lines. FAK phosphorylation at Tyr 397, 861 and Ser 910 was detected in the adherent cell line, Hep G2, however, U-937 showed weak FAK phosphorylation at Tyr 397 and Ser 910. This indicates that the prominent phosphorylation of FAK at Tyr 397, 861 and Ser 910 in Hep G2 but not in U-937 came as a result of the central role of FAK-integrin and FAK-Src associations in FAK phosphorylation at different residues.

Key words: Hep G2, U-937, anchorage-dependent growth, anchorage-independent growth

INTRODUCTION

Focal Adhesion Kinase (FAK) is a protein tyrosine kinase which is localized to focal adhesions (Schaller *et al*., 1992). FAK-integrins interaction stimulates FAK autophosphorylation at Tyr 397, this causes subsequent phosphorylation of other residues leading to FAK activation (Cox *et al*., 2006; Mitra and Schlaepfer, 2006; Mon *et al*., 2006). Previous reports have demonstrated FAK phosphorylation in the regulation of different cellular activities such as cellular growth, migration and adhesion (Schlaepfer *et al*., 1999; Ayaki *et al*., 2001). Cancer cell adhesion to extracellular matrix (ECM) is highly required to strengthen adhesion and to protect cells from undergoing anoikis (Schlaepfer *et al*., 1999). In addition, FAK has been implicated in protecting ECM-detached cells from undergoing apoptosis (Cance *et al*., 2000; Haier and Nicolson, 2000; Schaller, 2001). Several studies have demonstrated FAK role in several cancers, particularly in adherent cells derived from solid tumours (Ayaki *et al*., 2001; Suzuki *et al*., 2003; Fujii *et al*., 2004; Von Sengbusch *et al*., 2005). However, FAK expression and phosphorylation have been inadequately studied for non-adherent cancer cells.

FAK has been implicated in the differentiation of human myeloid cells at early stages of maturity (Recher *et al*., 2004). In past study (Kume *et al*., 1997), murine immature myeloid precursors exhibited low FAK levels. Furthermore, other studies have demonstrated FAK expression in neutrophils but not in CD34+cells and macrophages (Lévesque and Simmons, 1999; Fuortes *et al*., 1994; Fernandez *et al*., 1997). Our study was performed to examine FAK expression and phosphorylation in hepatocellular carcinoma cell line, Hep G2, as an example of adherent cancer cells, in the same way, leukemic cell line, U-937, was used as a non-adherent cancer cells. Apart from that, the sucellular localizations of total FAK and phosphorylated FAK proteins were also examined in Hep G2.

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In reference to these study findings, FAK expression was detected for both Hep G2 and U-937 cell lines. However, the phosphorylated FAK residues were predominantly observed in Hep G2. In this article, we discuss the current concept concerning the expression patterns and the importance of FAK phosphorylation in adherent-dependent and adherent-independent malignancies.

MATERIALS AND METHODS

Reagents: Antibodies used were as follows: Anti-FAK (total, phospho- Tyr 397, phospho-Tyr 407, phospho-Tyr 861, phospho-Ser 732, phospho-Ser 910) were all purchased from Upstate (Temecula, USA), HSC 70 and Alexa fluor 488 were purchased from Invitrogen (Paisley, UK). HRP-conjugated IgG goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Thermo Scientific (Rockford, USA). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Cell culture: Hepatocellular carcinoma and leukemic cell lines (Hep G2 and U-937 respectively) were purchased from American Type Culture Collection (ATCC, USA). MEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) was used to maintain Hep G2 cells. U-937 cells were cultured in RPMI-1640 (Invitrogen, Paisley, UK) supplemented with 10% FBS. Cells were grown in culture flasks and incubated at 37°C and 5% CO_2. The cell culture work was conducted at the Experimental Medicine and Advanced Natureceutical Testing and Research Laboratory (EMAN), School of Pharmaceutical Sciences, Universiti Sains Malaysia, Malaysia, in over the period June 2008 to November 2009.

Western blotting: Confluent Hep G2 cells were lysed in ice-cold cell extraction buffer (Biosource, Nivelles, Belgium) containing 1% protease inhibitor cocktail set 1 (Upstate, Temecula, California) and 0.1% phenylmethanesulfonyl fluoride (Sigma). Protein concentrations were determined using Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, USA). Proteins were resolved by SDS-PAGE using 8% gels. Resolved proteins were transferred into nitrocellulose membranes (Amersham Biosciences, UK) and subsequently probed with antibodies that recognise total and activated forms of FAK. Membranes were subjected to ECL detection reagent (Amersham Biosciences, Germany), bands were detected with LAS-3000 Luminescent Image Analyzer. This study Western Blotting analysis was carried out at the Laboratory of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Malaysia, in over the period June 2008 to November 2009.

Cells immunofluorescence: Hep G2 cells were grown on Poly-L-Lysine (Sigma) pre-coated coverslips. Cells were fixed with para formaldehyde (Sigma) and permeabilised using 0.1% Triton-X (Sigma). Samples were washed in TBS and blocked with TBS containing 1% BSA, 0.1% sodium azide for 30 min at RT. In a humidified chamber, samples were incubated with 200 μL of primary antibodies of anti-FAK antibodies (total, phospho-Tyr 397 and phospho-Tyr 861) at 1:250 dilutions for 1 h at RT. Samples were washed with TBS and probed with 200 μL of Alexa Flour® 488 at a dilution of 1:100 for 30 min at RT. Samples were co-stained for actin cytoskeleton with phalloidin (Molecular Probes, Invitrogen) and for nuclei with DAPI (Molecular Probes, Invitrogen) at a dilution of 1:40 and 1:35000 for 3 and 20 min, respectively at RT. Samples were mounted with ProLong® Gold Antifade Reagent (Molecular Probes, Invitrogen). Negative controls were prepared by substituting serum for primary antibody and no detectable staining was evident. The Immunofluorescence work was performed at the Institute of Pharmaceuticals and Nutraceuticals, Universiti Sains Malaysia, Malaysia, in over the period March 2008 to August 2009.

Microscopy: Cells were examined by phase contrast-fluorescence microscope (Olympus 1×71, Japan). Images were captured by OSIS XC50 camera (Olympus, Japan) under×400 magnification.

RESULTS

Total FAK and phospho-FAK Western blotting analysis: Total FAK was obviously detected in both Hep G2 and U-937 cell lines, while phospho-FAK Tyr 397 was comparably expressed in the cell lines. Since phospho-FAK Tyr 397 was clearly detected in Hep G2 the protein was weakly detected in U-937 cells. Neither Hep G2 nor U-937 revealed any expression of phospho-FAK Tyr 407 protein. Hep G2 expressed weak phosphorylation of FAK Tyr 861, while U-937 was deficient to phospho-FAK Tyr 861 expression.

In the present study, attention has not been confined to FAK phosphorylation at tyrosine sites, that FAK phosphorylation at serine residues was also examined. Here, none of the cell lines expressed phospho-FAK Ser 732 (Fig. 1). On the contrary, phospho-FAK Ser 910 was intensively expressed in Hep G2 but was undetectable in U-937.

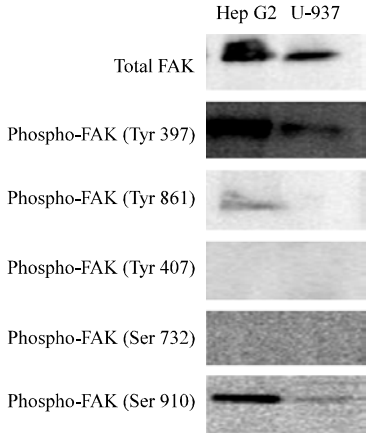

Fig. 1: Total FAK and phosphorylated FAK residues western blotting analysis in Hep G2 and U-937 cell lines. Equal amounts (30 μg) of lysate samples were separated through 8% SDS-PAGE gel electrophoresis. Separated proteins were transferred into nitrocellulose membranes and immunoblotted with specific antibodies against total FAK and phosphorylated FAK residues

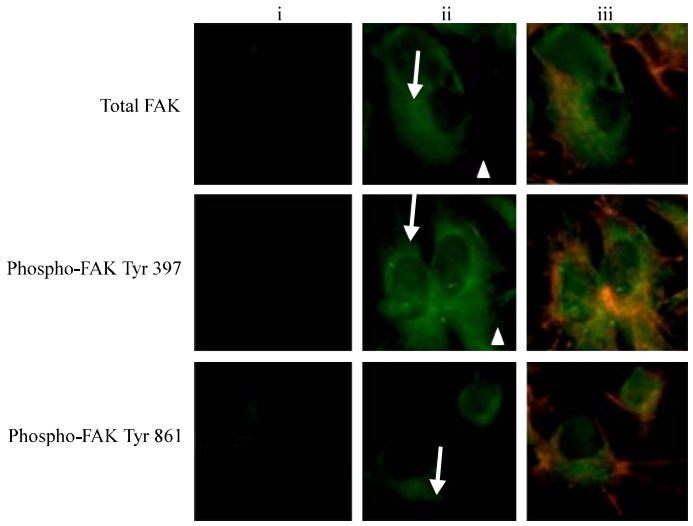

Fig. 2: Subcellular distribution of total FAK, phospho-FAK Tyr 397 and phospho-FAK Tyr 861 in Hep G2 cells. Cells were grown on coverslips, fixed and immunostained with antibodies recognise FAK and phosphorylated forms (Tyr 397 and 861) and secondary probed with Alexa fluor-488 conjugated antibody (green). Actin cytoskeleton was visualized using rhodamine stain (red), while nuclei were counter stained blue with DAPI stain. Negative control image (i). FAK subcellular localization (ii). Composite staining image of FAK, actin cytoskeleton and nuclei (iii). Arrow indicates cytoplasmic staining, while arrow head indicates staining at protrusions Magnification: x400

Cells immunofluorescence: With concern to protein subcellular distribution, few studies have discussed FAK and phosphorylated FAK residues localization to cytoplasm and cell protrusions in Hep G2. Given that our study seek to attain distinguishable expression patterns of FAK and phosphorylated FAK sites for adherent and non-adherent cells, scrutinizing protein subcellular localization of adherent cells will be worthwhile. In this study, indirect immunofluorescence analysis was performed to study the localization of total FAK protein, phospho-FAK Tyr 397 and phospho-FAK Tyr 861 in Hep G2. Subcellular localization of other forms of phosphorylated FAK protein was not done because of unavailability of specific antibody for this application.

Figure 2 articulates FAK and phosphorylated FAK immunofluorescent staining in Hep G2. Intense FAK staining was observed in cell cytoplasm with extended localization to cellular protrusions. However, phospho-FAK Tyr 397 staining was obviously detected for both cytoplasm and cellular protrusions. Phospho-FAK Tyr 861 staining was not clearly detected in the cell line.

DISCUSSION

Our study was able to delineate a preliminary interpretation about the necessity of FAK phosphorylation in adherent and non-adherent cancers. At early stages of blood tumourigenesis adhesion to marrow stromal cells protects AML cells from undergoing apoptosis and prolongs survival (Burger *et al*., 2003). FAK has been involved in the progression of blood diseases and in the relocation of AML cells from bone marrow to blood circulation (Recher *et al*., 2004; Tavernier-Tardy *et al*., 2009). In view of the fact that in comparable to adherent cancer cells FAK phosphorylation is not well examined in non-adherent cancers, this study aimed to study FAK expression and phosphorylation in hepatic and leukemic cancers.

Based on present study results (Fig. 1), we suggest that FAK expression and phosphorylation in Hep G2 support previously indicated role of FAK in adhesion-mediated downstream signalling of hepatocellular carcinoma (Von Sengbusch *et al*., 2005; Ren *et al*., 2008; Yam *et al*., 2009). In an adhesion-dependent signalling integrins engagement to ECM recruits signalling molecules to the site of focal adhesion (Michael *et al*., 2009) and triggers FAK autophosphorylation at Tyr 397 (Katz *et al*., 2002). Besides that, FAK phosphorylation at Tyr 397 has been proposed recently to FAK-Src association and phosphorylation of different FAK residues (Katz *et al*., 2002; Lietha *et al*., 2007). Therefore, we suggest that FAK phosphorylation at Tyr 397, Try 861 and Ser 910 in Hep G2 (Fig. 1) could happen as in adherent-dependent cancers FAK-integrin and FAK-Src associations triggered FAK phosphorylation at different residues. Present study findings (Fig. 2) where FAK, phospho-FAK Tyr 397 and phospho-FAK Tyr 861 were localized to Hep G2 came in consistence with other studies (Sein *et al*., 2000; Hauck *et al*., 2000; Miyazaki *et al*., 2003; Hsia *et al*., 2003; Mon *et al*., 2006) which correlated FAK localization to cellular protrusions to maintain adherent cancers aggressive phenotypes.

FAK has been implicated in the development of leukemic diseases, particularly at early stages, as it contributes to myeloid leukemia tumourigenesis. However, the concurrent expressions of FAK and phospho-FAK Tyr 397 were observed in marrow blast cells but not in mature AML cells (Recher *et al*., 2004; Tavernier-Tardy *et al*., 2009). Present study was able to recognize total and activated FAK proteins (mainly Tyr 397 and Ser 910) in U-937. Given that FAK has been implicated in prolonging survival of malignant cells in regardless of adhesion status (Haier and Nicolson, 2000; Zhao *et al*., 2001; Howe *et al*., 2002; Recher *et al*., 2004) we suggest that FAK expression and phosphorylation at Tyr 397 and Ser 910 are important to maintain U-937 survival.

In summary, our observations raise the possibility that FAK phosphorylation in adherent-dependent cancer cell line, Hep G2, is a function of adhesion. However, the weak expression of the phosphorylated FAK at Tyr 397 and Ser 910 in the adherent-independent cell line, U-937, could happen as the cell line is a floating cell. In other words, FAK is deficient to integrins, Src and ECM proteins associations. Our study puts forward a possibility that the weak or aberrant FAK expression and phosphorylation in the leukemic cell line happen as a result of the non-adhesive characteristics of the cell.

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