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Fibronectin enhances viability and alters cytoskeletal functions (with effects on the phosphatidylinositol 3-kinase pathway) in small cell lung cancer

T. Kijima [a], G. Maulik [a], P. C. Ma [a,b], Priya Madhiwala [a], E. Schaefer [c], R. Salgia [a] *

[a] Department of Medical Oncology, Dana-Farber Cancer Institute, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA,
[b] Department of Hematology and Oncology, New England Medical Center, Boston, MA,
[c] Biosource International, Hopkinton, MA

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Abstract

Small cell lung cancer (SCLC) is a rapidly progressive disease with ultimate poor outcome. SCLC has been shown to interact closely with the stromal and extracellular matrix (ECM) components of the diseased host. ECM consists of type I/IV collagen, laminin, vitronectin, and fibronectin (FN) among others. Herein, we investigated the behavior of a SCLC cell line (NCI-H446) on FN-coated surface. Over a course of 72 h, FN (10 μg/ml) caused both increased survival and proliferation of NCI-H446 cells. Survival under serum-starved conditions increased 1.44-fold and proliferation in the presence of fetal calf serum increased by 1.30-fold. The phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 reduced both survival and proliferation of NCI-H446 cells (0.48- and 0.27-fold, respectively), even on FN-coated surface. We next determined the effects of FN on cytoskeletal function such as cell motility/morphology and adhesion. Over a course of 24 h, FN reduced aggregation of NCI-H446 cells and induced flattened cellular morphology with neurite-like projections after 1 h, however, in the presence of LY294002, the cells rounded up. Adhesion of NCI-H446 cells also increased with FN (4.47-fold) which was abrogated with LY294002 treatment. This correlated with phosphorylation of the cytoskeletal protein p125FAK, on Tyr397, Tyr861 and Ser843 residues with FN. Even in the presence of LY294002, these serine/tyrosine residues were still phosphorylated on FN-coated surface. In contrast, the focal adhesion protein paxillin was not phosphorylated at Tyr31 with FN. In summary, FN stimulation of SCLC cells leads to enhancement of viability and changes in cytoskeletal function that are partially mediated through the PI3-K pathway.

Keywords: PI3-K - cell motility - cytoskeletal protein - extracellular matrix - small cell lung cancer

Introduction

Lung cancer has become a worldwide problem for which new therapeutics are desperately needed. In order to arrive at better therapies, we have to understand the tumor biology better. The interaction between the ECM/stromal components and lung cancer cells offers a unique opportunity to study how migration and metastasis of lung cancer cells occur. SCLC is the end-stage of a multi-step process of carcinogenesis, including deletion of tumor

* Correspondence to: Ravi SALGIA, MD
Dana-Farber Cancer Institute, Dana 1234B, 44 Binney Street, Boston, MA 02115, USA. Tel.: 617-632-3468, Fax: 617-632-4379, Email: ravi_salgia@dfci.harvard.edu

suppressor genes such as on chromosome 3p, p53 and Rb, and overexpression of oncogenes such as Myc and Bcl-2 [1]. The major cause of death from this disease is the associated metastasis.

Metastasis is a multi-step process which initially involves interaction between the SCLC cells and stroma. The stroma can modulate the motility and migration of SCLC cells. Integrins are involved in facilitating cell-cell adhesions and cell-matrix interactions [2]. SCLC cells are enveloped in the stroma of ECM, and it is thought that its high metastatic propensity and resistance to chemo- or radio- therapy arises from β1 integrin activation by the ECM proteins [3]. Metastasizing SCLC cells have a decreased level of F-actin, which is present and around the periphery of the cell. SCLC cells predominantly express the β1, α1, α2, α3, α6 and αv integrin chains, all of which are found also on normal bronchial epithelial cells, and have been shown to interact with laminin through α3β1 and α6β1 integrins [4]. SCLC cells possess low activation integrins that have a low affinity for ligands and are responsible for the detachment and motility of tumor cells [5]. Metastatic models have shown that transformation from the high to low activation state causes cells to break away from the ECM and migrate [6]. The signaling pathways involved are not yet clearly understood, however the RAS family of small GTP-binding proteins is implicated. Unlike in non-SCLC, RAS is not mutated in SCLC, but an immediate downstream effector, PI3-K, is constitutively activated in SCLC and may be responsible for cellular migration [5].

The interaction of tumor cells with the ECM not only can lead to altered adhesion but also altered cell motility. It has been shown that interaction with a variety of ECM surfaces can phosphorylate downstream signal transduction molecules in the cytoskeleton such as tensin, vinculin, p125FAK and paxillin [7]. p125FAK and paxillin have been repeatedly shown to be involved in mechanisms of migration of a variety of cells. p125FAK consists of an N-terminus integrin binding site, a central kinase domain, and C-terminus focal adhesion targeting and paxillin-binding domains; it is activated by tyrosine phosphorylation [8]. Through an autophosphorylation site at tyrosine 397, p125FAK can interact with several molecules, such as integrins, focal adhesion proteins, PI3-K, SH3 domain containing adapter proteins, and tyrosine kinases [8]. Paxillin is a 68 kDa protein with four tandem LIM domains and several serine/tyrosine phosphorylation sites, where Crk/CRKL, Src, and other proteins can bind, and the protein itself can associate with certain oncogene products [9]. Tyrosine residues at amino acid 31 and 118 are important in binding to adapter protein CRKL [10].

In this study, we have determined the behavior of NCI-H446 SCLC cell line altered with FN in terms of viability, morphology, and adhesion. We have also determined the effects of inhibiting the PI3-K pathway on NCI-H446 cells. Finally, we show the differential phosphorylation on p125FAK and paxillin with FN in the context of PI3-K.

Materials and methods

Cell line and cell culture

The SCLC cell line NCI-446 was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Cellgro) supplemented with 10% (v/v) fetal calf serum (FCS). In some experiments, cells were treated with PI3-K inhibitor LY294002 (20 μM) (Sigma, St. Louis, MO).

Cell viability assay

The wells of a 96-well tissue culture plate (Corning-Costar, Cambridge, MA), precoated with 10 μg/ml human plasma FN (Life Technologies, Inc., Rockville, MD) overnight at 4 ºC, were washed with PBS twice and blocked for 1 h at 37ºC with RPMI 1640 medium containing 0.5% (w/v) BSA (Sigma) before plating cells. NCI-H446 cells (1×10^5 for survival and 1×10^4 for proliferation, respectively) were plated onto untreated or FN-treated surface, then cultured with or without LY294002 (20 μM) in serum free (0.5% BSA) or serum containing (10% FCS) media. After 72 h incubation, the relative number of viable cells was analyzed and quantified by the MTT colorimetric assay (Sigma) following the instruction manual. Student's *t* test was used for the statistical analysis and differences were considered statistically significant at $P < 0.05$.

Adhesion assay

Serum starved NCI-H446 cells (1 x 10^5) were plated onto untreated or FN (10 μg/ml)-treated surface in serum free (0.5% BSA) media. After 6 h incubation with or without LY294002 (20 μM) at 37ºC, unattached cells were removed by gentle washing with PBS. The relative number of attached viable cells was determined by the MTT colorimetric assay. Student's *t* test was used for the statistical analysis of the attached cell number, and differences were considered significant at $P < 0.05$.

Cell motility/morphology analysis

NCI-H446 cells (1 x 10^6/ml) were plated onto FN (10 μg/ml)-coated or uncoated 35mm-diameter dish and cultured in serum free condition. Phase-contrast pictures (20x) were taken using an Olympus IX70 inverted microscope at indicated time points. To examine the effects of PI3-K inhibitor on cell motility, NCI-H446 cells (1 x 10^4 without compounds and 1 x 10^5 with 20 μM LY294002, respectively) were cultured on untreated or FN-treated surface of the wells of a 96-well tissue culture plate with 10% FCS for 48 h, then phase-contrast pictures (10x) were taken and shown.

Antibodies

The anti-phosphotyrosine monoclonal antibody 4G10 and anti-p85 PI3-K polyclonal antibody were obtained from UBI (Lake Placid, NY). The anti-p125FAK polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-paxillin monoclonal antibody (clone 5H11), and phosphorylation site-specific polyclonal antibodies (Biosource International, Camarillo, CA); phospho-FAK [tyrosine 397 (autophosphorylation site) and 861, and serine 843] and phospho-paxillin [tyrosine 31 (CRKL SH2 domain binding site)] antibodies were used as described previously [11, 12].

Preparation of cell lysates and immunoblotting

Cells were lysed in lysis buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, and 0.42% NaF] containing inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Cell lysates were separated by 7.5% SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Proteins were detected by immunoblotting using an enhanced chemiluminescense technique (NEN Life Science Products, Boston, MA). Also immunoprecipitations were performed according to standard procedures and immunoblotting thereafter [11, 13].

Results

FN surface increases survival and proliferation of NCI-H446 SCLC cells through PI3-K

Interaction with the ECM can be important for the metastatic potential of the tumor cell. We examined whether FN can enhance the viability of SCLC cells in terms of survival and proliferation. Over the course of 72 h, FN significantly increased not only cell survival (1.44-fold), measured in the absence of FCS (Fig. 1A), but also proliferation (1.30-fold) in serum containing condition (Fig. 1B) of NCI-H446 SCLC cells. The PI3-K inhibitor LY294002 (20 μM) decreased both survival and proliferation of NCI-H446 cells (0.48- and 0.27-fold, respectively). This could not be overcome by stimulation of cells on a FN-coated surface, suggesting that PI3-K plays an important role in regulating these events.

FN induced cell motility/morphology and adhesion of NCI-H446 SCLC cells through PI3-K

We next determined the effects of FN on cytoskeletal function such as cell motility/morphology and adhesion. Over the course of 24 h, most of the cells kept rounded shape, formed clusters after 1h, and weakly attached to untreated surface. On the other hand, FN reduced the characteristic aggregation of NCI-H446 cells and most of the cells formed flattened shape with neurite-like projections after 1 h (Fig. 2). Within 48 h of FN treatment, even in the presence of FCS, morphologic changes observed as in Fig. 2 were similar as shown in Fig. 3c as com-

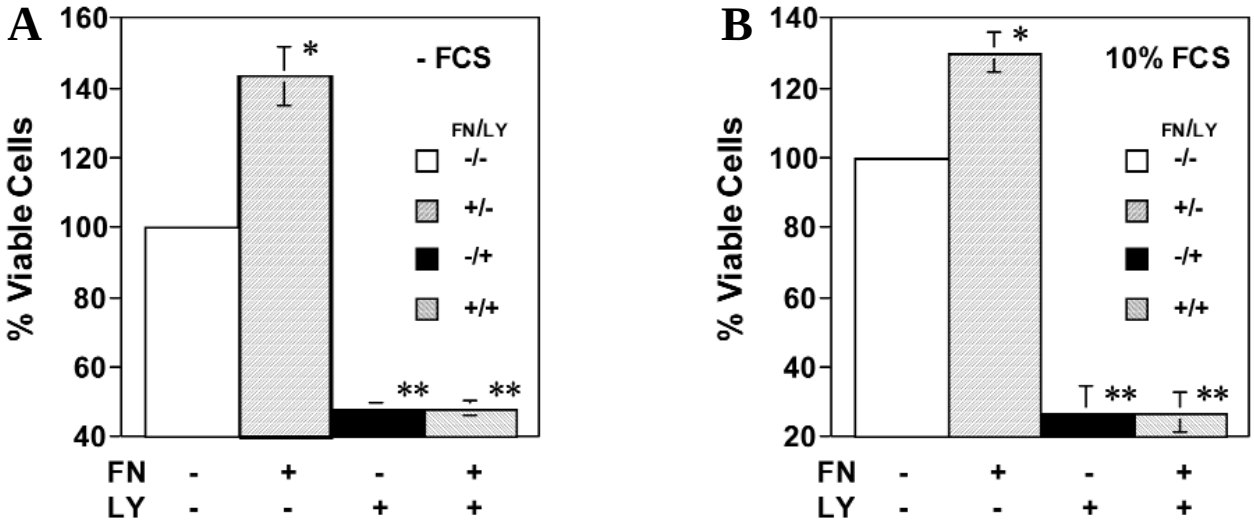

Fig. 1 FN induces both survival and proliferation of SCLC cells through PI3-K. NCI-H446 cells were plated onto untreated or FN (10 μg/ml)-treated surface then cultured with or without LY294002 (20 μM) in serum free (0.5% BSA) (A) or serum containing (10% FCS) (B) media. Number of viable cells was analyzed and quantified by MTT colorimetric assay after 72 h incubation. Relative percentage of viable cells was shown as mean (columns) ± SD (bars) from three independent experiments. FN significantly induced not only cell survival (1.44-fold) when starved (A) but also proliferation (1.30-fold) in serum containing condition (B). LY294002 reduced cell viability (0.48- and 0.27-fold, respectively) even on FN-coated surface. *; P =0.0008, **; P <0.0001, statistically significant compared with untreated condition.

pared to without FN treatment (Fig 3a). Inhibition of PI3-K with LY294002 lead to rounded morphology of the cells (Fig. 3b and 3d).

We also determined the effects of FN on cell adhesion. FN also increased cell attachment of NCI-H446 cells (4.47-fold, P =0.0015). Consistent with the above experiments, LY294002 decreased the number of attached cells 0.36-fold (P =0.0025) onto untreated surface and 0.35-fold (P =0.0039) even on FN-coated surface (Fig. 4). This would again implicate the PI3-K pathway being crucial in cytoskeletal functions in SCLC.

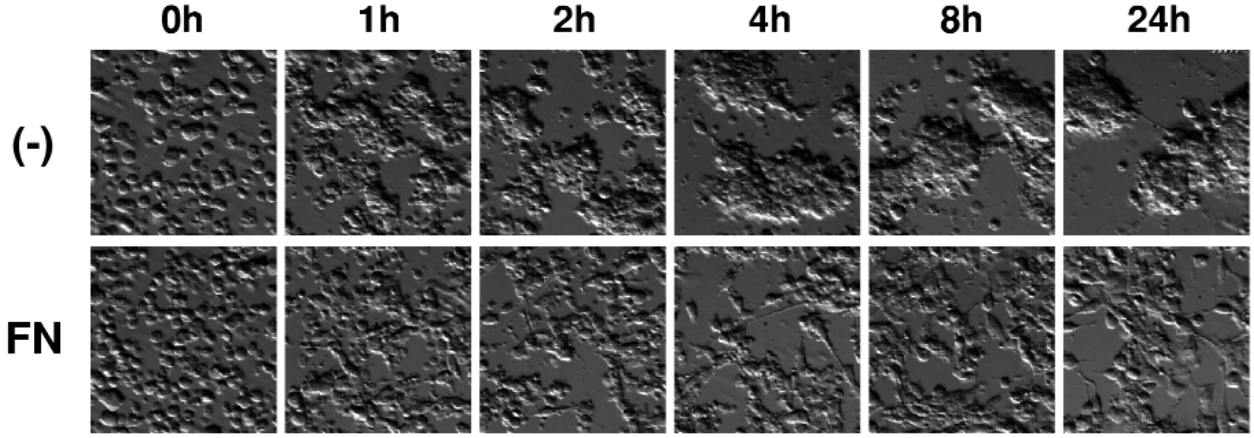

Fig. 2 FN induces morphological changes in SCLC cells. NCI-H446 cells (1x10^6/ml) were plated onto FN (10 μg/ml)-coated or uncoated surface and cultured in serum free condition. Phase-contrast pictures (20x) were taken and shown at indicated time points. Most of cells remained rounded in shape, formed clusters after 1h, and weakly attached to untreated surface. On the other hand, cells on FN-coated surface were much more tightly adherent without clump formation, became flattened with cell spreading, and formed neurite-like projections after 1h.

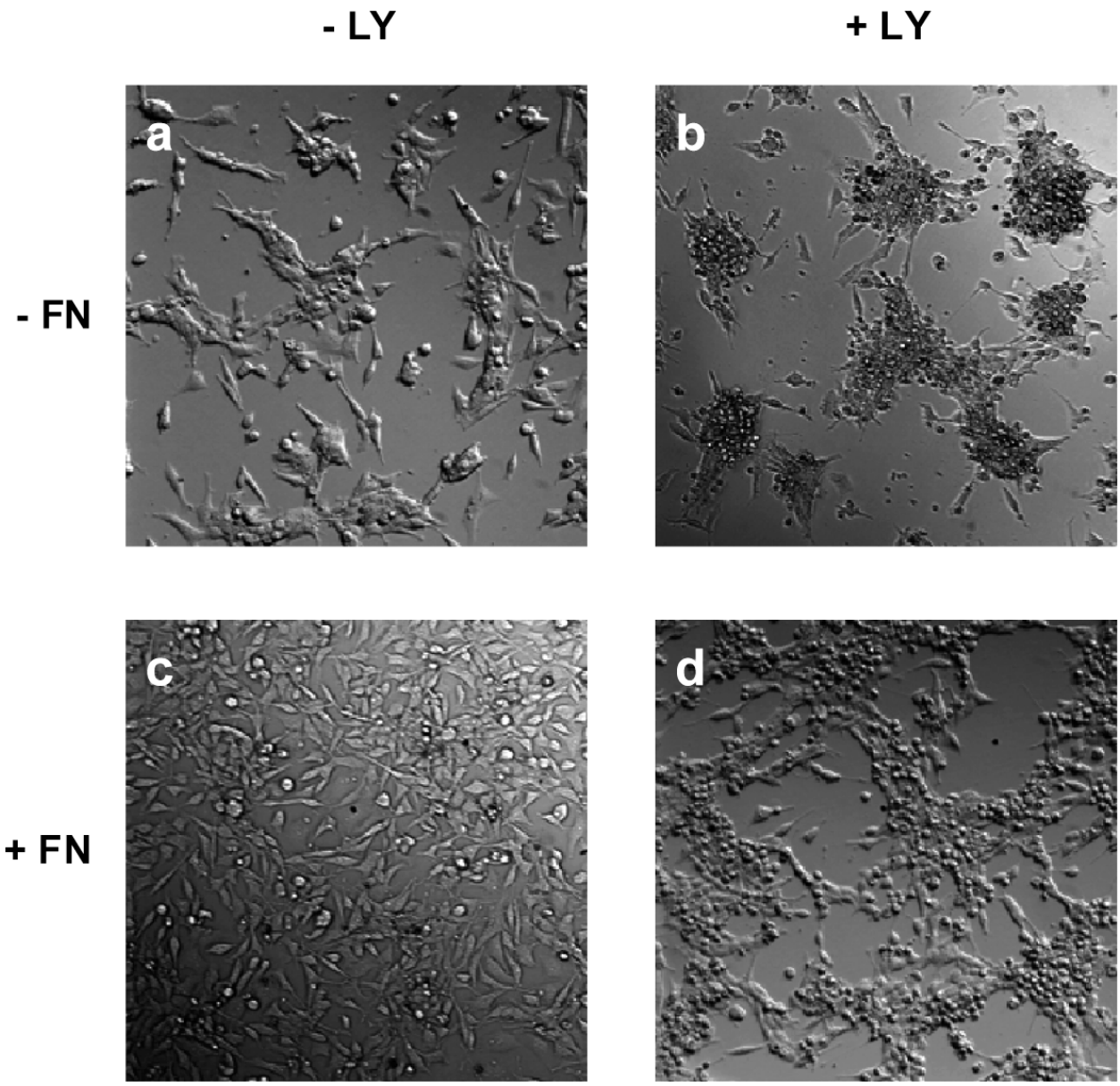

Fig. 3 PI3-K regulates FN-induced cell motility in SCLC cells. NCI-H446 cells, $1x10^4$ without compounds and $1x10^5$ with LY294002 (20 μM) were cultured on untreated or FN (10 μg/ml)-treated surface in serum containing media for 48 h then phase-contrast pictures (10x) were taken and shown. (a) Without FN, cells adhered and formed projections. Some of them gathered to make small clumps but cell number was low. (b) In the presence of LY294002, most cells formed clusters with projections and looked apoptotic with small and rounded shape. (c) On the other hand, on FN-coated surface, cell number apparently increased and each cell tightly adhered separately then became flattened with projections. (d) LY294002 abrogated this phenomenon leading cells to apoptosis.

FN regulates phosphorylation of p125FAK

We next determined the biochemical consequences of FN stimulation in NCI-H446 cells. Several tyrosine phosphorylated bands were identified around 130-145, 115-125, 85-95, and 60 kDa by FN stimulation. LY294002 decreased these FN-induced tyrosine phosphorylations (Fig. 5A). We identified p125FAK as a prominent target of tyrosine phosphorylation in FN-stimulated NCI-H446 cells. p125FAK was phosphorylated on Tyr397, Tyr861 and Ser843 by FN. However, in the pres-

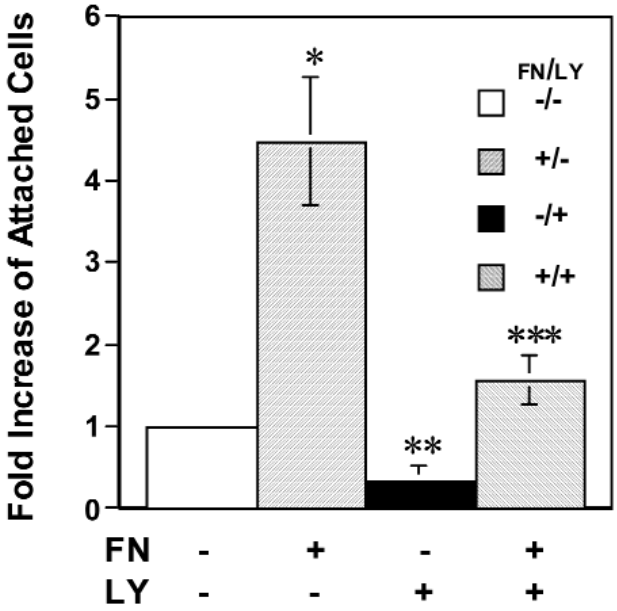

Fig. 4 FN induces adhesion of SCLC cells through PI3-K. Serum starved NCI-H446 cells ($1x10^5$) were incubated for 6 h at 37 °C on untreated or FN (10 μg/ml)-treated surface with or without LY294002 (20 μM). Relative number of attached viable cells was determined by MTT colorimetric assay and shown as mean (columns) ± SD (bars) fold increase from three independent experiments. FN increased cell attachment 4.47-fold (P =0.0015). LY294002 decreased the number of attached cells 0.36-fold (P =0.0025) on untreated surface and 0.35-fold (P =0.0039) even on FN-coated surface. *; P =0.0015, **; P =0.0025, compared with untreated condition, ***; P =0.0039, compared with FN-treated condition.

ence of LY294002, differences in the phosphorylation of these amino acid residues in p125FAK with FN were not appreciated (Fig. 5B). In contrast, paxillin on Tyr31 was not phosphorylated in any of these conditions (Fig. 5C).

Discussion

SCLC is a unique illness that is defined by its aggressive nature with frequent metastases. In order to metastasize, SCLC cells have to interact with the ECM components. In this study, we have shown that FN causes increased survival and proliferation of NCI-H446 cells. Furthermore, FN stimulates cell motility, migration, and adhesion of the SCLC cells. All of these biological functions are inhibitable by LY294002, a specific inhibitor of the PI3-K pathway. Interestingly, the downstream target of FN stimulation, specifically cytoskeletal protein p125FAK, in this model does not seem to be inhibited via the PI3-K pathway inhibition.

PI3-K is a heterodimer composed of p85 regulatory and p110 catalytic subunits. This family of enzymes is responsible for cellular regulation, including such events as proliferation, reduced apoptosis, anchorage independence, and intracellular vesicle trafficking/secretion [14, 15]. We have further shown that PI3-K is a crucial pathway in biological functions stimulated by FN in SCLC. Although the basal activity rate of PI3-K in SCLC is high, the mechanisms of activation have not been deciphered [16]. However, suggested modes include the recruitment of the SH2 domain of p85 PI3-K to a phospho-Tyr-X-X-Met motif by growth factor receptors or ECM components, inducing the movement of the regulatory p85 PI3-K to the membrane [5]. We have also recently shown that PI3-K can be activated via activating c-Kit and c-Met receptor tyrosine kinases, as well as activating CXCR4 chemokine receptor [11, 13, 17]. It would be useful for the future to elucidate what role receptor tyrosine kinases, chemokine receptors, and ECM components have in activating biological functions of SCLC as well as effects on downstream targets such as PI3-K.

The role of the ECM and its interaction with tumor cells is complex and here we have examined this specifically in the context of SCLC. The ECM consists of a basement membrane and its interstitial stroma, both of which are composed of proteoglycans and collagens [5]. The matrix itself consists of collagen, FN, fibril-associated proteoglycans, and hyaluronic acid, while the basement membrane is made of type IV collagen, laminin, and also proteoglycans [5]. SCLC cells, *in vivo*, are bound at both primary and secondary sites by a stroma of FN, type IV collagen, and laminin, which has the ability to stimulate cell migration, adhesion, and metastases [18]. SCLC cells attach to the ECM components via integrins.

Integrins are cell-surface receptors that allow for adhesion to ECM proteins and other cells [2]. They are composed of two different types of chains, α and β, that associate into heterodimers. They are significant for their role in cellular proliferation,

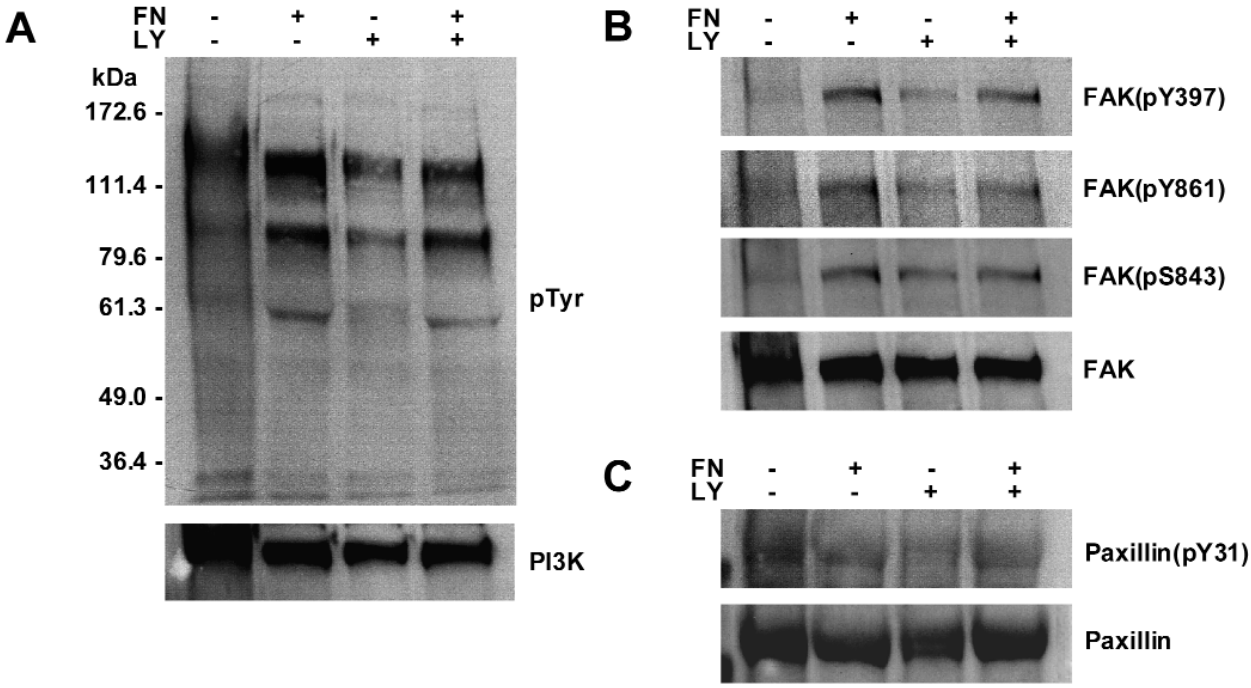

Fig. 5 FN phosphorylates p125FAK, independent of PI3-K, but not paxillin in SCLC cells. NCI-H446 cells, pre-treated with or without LY294002 (20 μM) for 2 h, were plated onto untreated or FN (10 μg/ml)-treated surface and incubated in serum free media before lysis. Whole cell lysates or samples immunoprecipitated with anti-p85 PI3-K antibody were applied to 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-phosphotyrosine (4G10), p85 PI3-K, p125FAK, phospho-FAK (tyrosine residues 397 or 861 and serine residue 843), p68 paxillin, and phospho-paxillin (tyrosine residue 31) antibodies. (A) Several tyrosine phosphorylated bands were identified around M_w 130-145, 115-125, 85-95, and 60 kDa by FN. LY294002 decreased these FN-induced phosphorylation. The membrane was stripped and re-blotted with anti p85 PI3-K as a control. (B) FN phosphorylated p125FAK at tyrosine 397 and 861 and serine 843 positions. However LY294002 could not reduce these FN-induced phosphorylations, though expression of total FAK was same in each condition. (C) FN did not phosphorylate paxillin at tyrosine 31.

motility, and metastasis [2]. Tumor cells tend to have a lower number of integrins than normal cells because of decreased differentiation of oncogenic transformation. SCLC cells predominantly express the β1, α1, α2, α3, α6 and αv integrin chains. SCLC cells possess low activation integrins that have a low affinity for ligands and are responsible for the detachment and motility of tumor cells [5]. Metastatic models have shown that transformation from the high to low activation state causes cells to break away from the ECM and migrate [6].

Cytoskeletal proteins and the actin cytoskeleton itself are involved in the regulation of cell motility, structure, and adhesion [7]. The actin cytoskeleton associates with the ECM through focal adhesion, which contains tensin and vinculin, both of which have tumor suppressor properties [7]. Disruption of the cytoskeleton causes invasion growth to occur, because SCLC cells characteristically are non-adherent and metastasize frequently [19]. Cancerous cells increase their movement along the basement membrane through disturbance of the normal cytoskeleton [7]. As previously discussed, integrin-mediated transformations are directly involved in metastases and help in maintaining the integrity of the cytoskeleton. One of the key components for the cytoskeleton is p125FAK.

p125FAK, a 125 kDa protein, consists of an N-terminus integrin binding site, a central kinase domain, and C-terminus focal adhesion targeting and paxillin-binding domains, and it is activated by tyrosine phosphorylation [8]. Through an autophosphorylation site at tyrosine 397, p125FAK can interact with several molecules, such as integrins, focal adhesion proteins, PI3-K, SH3 domain containing adapter proteins, and tyrosine kinases [12,

20]. Studies have shown that over-expression of p125FAK in Madin-Darby canine kidney cells increases the cellular migratory behavior produced by hepatocyte growth factor/scatter factor (which stimulates the c-Met receptor tyrosine kinase). However, this effect is eliminated when FAK mutants that cannot bind PI3-K are produced [11, 12]. In our studies, we found that FN stimulation caused phosphorylation of the autophosphorylation site at tyrosine 397, src binding site at tyrosine 861, and the site important in mitosis at serine 843. Surprisingly, the phosphorylation with FN still occurred in the presence of the PI3-K inhibitor LY294002. So, even though we have had dramatic reduction of biological functions such as viability, cell motility, cellular migration and adhesion with LY294002, the downstream signal transduction to p125FAK still remains intact. This would implicate differential regulation of cytoskeletal function.

In summary, we have shown dramatic effects of fibronectin on viability and cytoskeletal functions through PI3-K pathway in SCLC. We have further shown that p125FAK is an important molecule in FN signaling for SCLC cells. It would be useful for the future to determine what role inhibiting the interaction between SCLC cells and the ECM would have in clinical therapeutics.

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