Inhibition of the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Pathway but not the MEK/ERK Pathway Attenuates Laminin-Mediated Small Cell Lung Cancer Cellular Survival and Resistance to Imatinib Mesylate or Chemotherapy

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Abstract

The fact that small cell lung cancer (SCLC) is commonly incurable despite being initially responsive to chemotherapy, combined with disappointing results from a recent SCLC clinical trial with imatinib, has intensified efforts to identify mechanisms of SCLC resistance. Adhesion to extracellular matrix (ECM) is one mechanism that can increase therapeutic resistance in SCLC cells. To address whether adhesion to ECM increases resistance through modulation of signaling pathways, a series of SCLC cell lines were plated on various ECM components, and activation of two signaling pathways that promote cellular survival, the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway and the mitogen-activated protein kinase kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway, was assessed. Although differential activation was observed, adhesion to laminin increased Akt activation, increased cellular survival after serum starvation, and caused the cells to assume a flattened, epithelial morphology. Inhibitors of the PI3K/Akt/mTOR pathway (LY294002, rapamycin) but not the MEK/ERK pathway (U0126) abrogated laminin-mediated survival. SCLC cells plated on laminin were not only resistant to serum starvation–induced apoptosis but were also resistant to apoptosis caused by imatinib. Combining imatinib with LY294002 or rapamycin but not U0126 caused greater than additive increases in apoptosis compared with apoptosis caused by the inhibitor or imatinib alone. Similar results were observed when adenoviruses expressing mutant Akt were combined with imatinib, or when LY294002 was combined with cisplatin or etoposide. These studies identify laminin-mediated activation of the PI3K/Akt/mTOR pathway as a mechanism of cellular survival and therapeutic resistance in SCLC cells and suggest that inhibition of the PI3K/Akt/mTOR pathway is one strategy to overcome SCLC resistance mediated by ECM. (Cancer Res 2005; 65(18): 8423-32)

Introduction

SCLC accounts for ~15% to 20% of all lung cancer cases in the United States and is typically characterized by initial therapeutic responsiveness followed by disease recurrence that is much more aggressive in nature and chemotherapeutically resistant (1). Over the last 20 years, only incremental improvement in survival has been noted with chemotherapy in SCLC, which has prompted clinical evaluation of more “targeted” drugs such as imatinib (2). Whereas the mechanisms by which SCLC cells acquire the ability to evade chemotherapy-induced death is unclear, composition of the tumor microenvironment, specifically extracellular matrix (ECM) composition, may play a role (3, 4).

ECM is composed of protein polymers that are organized into a meshwork (5). Cell adhesion to the ECM is mediated through the integrin family of heterodimeric transmembrane receptors, which are composed of α and β subunits (6). Most transformed cells (including most SCLC cell lines) have developed the ability to survive and proliferate in the absence of cell adhesion (7). Interestingly, SCLC cells in vivo are surrounded by a dense stroma of ECM at primary and metastatic sites (4). Adhesion of SCLC cells to ECM proteins in vitro confers resistance to chemotherapy-induced apoptosis that is dependent on protein tyrosine kinase activity (4). These data suggest that adhesion-dependent activation of signaling pathways may contribute to chemotherapeutic resistance in SCLC.

Of the pathways activated by integrin-ECM binding, the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathways are essential for integrin-mediated regulation of cell proliferation and survival (8, 9). Both pathways play key roles in tumorigenesis and therapeutic resistance (10, 11). Inhibition of the PI3K/Akt pathway sensitizes non-SCLC (NSCLC) or SCLC cells to traditional chemotherapeutic agents (12, 13). In addition, adherent SCLC cells that are selected after prolonged subculturing show increased activation of Akt and ERK and greater resistance to traditional chemotherapy or radiation (14). Enhanced survival of SCLC cells on physiologic ECM proteins may therefore be due to adhesion-dependent activation of the PI3K/Akt/mTOR and/or MEK/ERK pathways.

Here we report that SCLC cell lines differentially activate Akt and ERK activity after adhesion to specific ECM components. Adhesion to laminin increased Akt activity, altered cell morphology, and promoted resistance to serum withdrawal-induced cell death. Inhibitors of the PI3K/Akt/mTOR pathway but not MEK/ERK pathway abrogated laminin-mediated survival. Although SCLC cells...
adherent to laminin were unaffected by the addition of imatinib, biochemical or genetic inhibition of the PI3K/Akt/mTOR pathway induced responsiveness to imatinib, as well as traditional chemotherapies. These results suggest that adhesion-dependent activation of the PI3K/Akt/mTOR pathway may be one of the factors involved in the survival and chemotherapeutic resistance of SCLC. Targeting this pathway may be a useful approach to improve the therapeutic responsiveness of SCLC cells.

Materials and Methods

Cell culture. SCLC cell lines H69, H345, H209, H128, and H526 were obtained from Dr. Frederic Kaye (National Cancer Institute/Navy Medical Oncology) and were grown and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) plus 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin-streptomycin (Life Technologies). H510 cells were grown in Hite’s media (Life Technologies) plus 5% FBS and 1% penicillin-streptomycin at 37°C, 7.5% CO2. For low-serum conditions, the serum concentration was reduced to 0.1% FBS.

Reagents. Phospho-specific antibodies directed against Ser473 (S473) of Akt, Thr308 and Tyr320 (T202/Y204) of ERK-1/2, S389 of p70S6K, native antibodies against Akt, ERK-1/2, and p70S6K and antibodies against hemaglutinin, phospho-c-Kit (Y719), c-Kit, PDGFRα, PDGFRβ, and β-galactosidase (β-Gal) were purchased from Cell Signaling Technology (Beverly, MA). α-Tubulin antibodies were obtained from Sigma-Aldrich (Milwaukee, WI). The α1 integrin–activating antibody (TS2/16) was a gift of Dr. David Roberts (NIH, Bethesda, MD) and has been previously described (15). LY294002, rapamycin, and U0126 were purchased from Calbiochem EMD Biosciences, Inc. (La Jolla, CA). Imatinib was obtained from Novartis Pharmaceuticals Corp. (East Hanover, NJ). Six- and 12-well coated plates (laminin, fibronectin, and collagens I and IV) were purchased from Discovery Labware BD Biosciences (Bedford, MA). Stem cell factor (SCF) was purchased from Sigma-Aldrich, and platelet-derived growth factor (PDGF) was obtained from R&D Systems (Minneapolis, MN).

Extracellular matrix adhesion assays. All cell lines were plated on plastic or ECM-coated tissue culture plates (laminin, fibronectin, and collagens I and IV) in low-serum conditions and cultured for 5 days at 37°C, 7.5% CO2. After culturing, cells were harvested and processed for immunoblot analysis as described below. For time course experiments, H69, H209, H128, and H526 cells were plated on plastic or laminin-coated tissue culture plates in low-serum conditions and cultured for the indicated times followed by harvesting and processing for immunoblot analysis.

Stimulation with stem cell factor or platelet-derived growth factor. H69, H209, and H526 were serum starved overnight on 6-well plates in RPMI 1640 with low-serum conditions, and 50 ng/mL SCF or 100 ng/mL PDGF-AB were added 10 minutes before harvest. In the combinatory treatment with imatinib, the cells were pretreated with imatinib for 30 minutes before administration with SCF.

Apoptosis. For apoptosis experiments, H69 and H209 cells were plated on plastic or laminin-coated tissue culture plates in low-serum conditions and cultured for 3 days at 37°C, 7.5% CO2. Following adhesion, cells were treated or not with LY294002, rapamycin, or U0126 in the presence or absence of imatinib, and the cells were cultured for an additional 72 hours. After the total culture time (6 days), cells were harvested and processed for flow cytometric analysis of apoptosis, as assessed by formation of subgenomic DNA fragmentation (12).

Adenoviral infection of H69 cells. H69 cells were plated on plastic or laminin-coated tissue culture plates in low-serum conditions and cultured for 2 days at 37°C, 7.5% CO2. The cells were then infected with adenoviral particles encoding either the adenoviral-encoded βGal gene (Ad-βGal) or adenoviral-encoded dominant-negative Akt (Ad-dnAkt) for 24 hours. (These adenoviruses were kind gifts of Dr. Kenneth Walsh, Boston, MA). Following the infection period, the cells were treated or not with imatinib for 72 hours, after which the cells were harvested and processed for flow cytometric and immunoblot analysis as previously described (12).

Immunoblotting. Cell extracts were prepared for SDS-PAGE and immunoblot analysis as previously described (12).

Statistical analysis. Statistical comparison of mean values was done using the Student's t test. All P values are two tailed.

Results

Small lung cancer cell lines show differential activation of Akt and extracellular signal-regulated kinase in response to extracellular matrix adhesion. To determine the level of Akt activation in SCLC cells, we screened three SCLC cell lines (H69, H345, and H510) against a panel of NSCLC cell lines that are known to vary in constitutive Akt activity (12). When cells plated on plastic were serum deprived, the SCLC cell lines failed to exhibit Akt activation. Two NSCLC cell lines (H157 and H1155) showed high levels of endogenous Akt activation, consistent with earlier observations (ref. 12; Fig. 1A, top). In contrast to lack of Akt activation, two of three SCLC cell lines (H345 and H510) showed constitutive ERK activation when serum deprived (Fig. 1A, middle). To ensure that the lack of Akt activity in these SCLC cell lines was not due to an absence of functional Akt signaling pathways, SCLC cells were stimulated with insulin-like growth factor-I (IGF-I) under normal growth conditions or serum deprivation. IGF-I increased Akt and ERK activation in the three SCLC cell lines under each growth condition (Fig. 1B). Thus, the absence of Akt activity in SCLC cell lines under low-serum conditions is a result of serum deprivation and not due to deficiencies in Akt signaling pathways in these cells.

To examine whether specific ECM proteins altered Akt or ERK activation in SCLC cells, we assessed changes in Akt or ERK activation in H69, H345, and H510 cells plated on plastic (P), laminin (L), fibronectin (F), collagen I (C1), or collagen IV (C4), under normal growth conditions or serum deprivation. Under normal growth conditions, H69 and H510 cell lines increased Akt activation on all the ECM proteins tested, whereas the H345 cells exhibited increased Akt activation on all except collagen IV (Fig. 1C, left). With serum deprivation, H69 cells exhibited Akt activity on laminin, fibronectin, and collagen I, whereas H345 cells responded on laminin and fibronectin but not collagens I or IV (Fig. 1C, right). H510 cells did not increase Akt activation on any ECM protein (Fig. 1C, right). The different response of H510 cells may be attributed to the fact that this cell line was derived from an adrenal metastasis in a patient with SCLC (16); thus, these cells may respond differently to adhesion to different ECM proteins.

The MEK/ERK cascade promotes the survival of NSCLC cells and can be activated in response to cell adhesion (17). We therefore assessed activation of the MEK/ERK pathway in SCLC cell lines in response to ECM adhesion by monitoring the phosphorylation of ERK. Under high-serum conditions (Fig. 1C, left), H69 cells increased ERK phosphorylation on laminin and collagen IV, whereas H345 cells showed increased ERK phosphorylation on laminin and collagens I and IV. Under low-serum conditions (Fig. 1C, right), H69 cells only increased ERK activation when plated on laminin, whereas H345 cells did not activate ERK on any ECM protein. Levels of ERK phosphorylation were below baseline when H345 cells were plated on fibronectin or collagens I or IV. Interestingly, whereas the H510 cell line showed constitutive ERK activity when grown on plastic, these cells did not increase ERK activation on any matrix under either high- or low-serum conditions. Laminin or fibronectin inhibited ERK activation in these cells. Taken together, these data show that Akt and ERK are activated in SCLC cells in a cell line– and ECM-specific manner. Because laminin consistently increased Akt
and ERK activation in H69 cells, adhesion of these cells to laminin was explored further.

Adhesion of small cell lung cancer cells to laminin increases Akt and extracellular signal-regulated kinase activation and increases cell survival and alters cell morphology. To assess the time dependence of activation of Akt and ERK and to assess cellular behavior after adhesion to laminin, we assessed parallel samples on days 1, 3, and 5 for immunoblotting, flow cytometry, and phase-contrast microscopy. On day 1, phosphorylation of ERK but not Akt was increased in cells plated on laminin (Fig. 2A, top left). On days 3 and 5, the activation of Akt and ERK was increased in cells on laminin compared with plastic. Maximal activation of Akt and ERK was observed on day 5.

Increases in Akt and ERK activation in SCLC cells plated on laminin correlated with progressive changes in cellular morphology (Fig. 2A, bottom). H69 cells grown on plastic remained in suspension as large cell aggregates through the experiment. After 1 day on laminin, some of the H69 cells became adherent. By days 3 and 5, virtually all of the cells were attached and showed an adherent, flattened morphology. Similar changes in morphology
after attachment were also observed in the H345 and H526 cells (data not shown). Adhesion was not always accompanied by morphologic changes, however. H128 and H209 cells became completely attached within 12 hours of plating, but this was not accompanied by discernable morphologic changes (data not shown). When cellular survival in response to serum withdrawal was assessed at day 5, H69 cells plated on laminin showed almost 5-fold less apoptosis compared with cells grown on plastic (Fig. 2A, top right).

To generalize these observations, we characterized three additional SCLC cell lines. H209, H128, and H526 cells were grown on plastic or laminin under low-serum conditions, and time-dependent activation of signaling pathways on laminin or plastic was assessed. In H209 and H526 cells (Fig. 2B, top and middle, respectively), phosphorylation of Akt was increased on laminin on days 3 and 5. In H128 cells (Fig. 2B, bottom), basal levels of Akt phosphorylation were high on plastic or laminin and did not differ during the course of the experiment (Fig. 2B, bottom).
Phosphorylation of ERK diminished with time in H209 and H526 cells but was maintained to a greater extent in cells plated on laminin (Fig. 2B, top and middle). Similar induction of Akt activation on laminin was also observed in H209 and H526 cells when they were grown in medium containing 10% FBS (data not shown). These results suggest that laminin-stimulated activation of the PI3K/Akt or MEK/ERK pathway is a common event in the SCLC cells.

Cell adhesion to the ECM protein laminin has been shown to be mediated by β1-containing integrin receptors in other cell types (18). To determine if β1 integrin receptors were involved in the laminin adhesion–dependent activation of Akt in H69 cells, we evaluated activation of Akt and ERK in response to β1-activating antibodies when cells were plated on plastic (Fig. 2C). These antibodies have been previously shown to specifically induce β1 activity (15). Whereas no difference in Akt activation between β1- and control-treated H69 cells was observed on day 1, increased activation of Akt in response to β1 activating antibodies compared with control IgG was observed on day 3. By day 5, Akt activity decreased below baseline for both conditions. Compared with control IgG, ERK activation was dramatically increased at day 1 in response to the β1-activating antibody. On days 3 and 5, however, control IgG nonspecifically increased ERK phosphorylation, which limits interpretation of these results. Nonetheless, these results show that activation of Akt and ERK is increased in a time-dependent manner in response to SCLC cell adhesion to laminin, and this is partially recapitulated by treating SCLC cells on plastic with β1 integrin–activating antibodies.

Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway attenuates laminin-mediated protection of small cell lung cancer cells and induces responsiveness to imatinib. To evaluate the contribution of the PI3K/Akt/mTOR or MEK/ERK pathways to laminin-mediated survival, the ability of kinase inhibitors to decrease protection conferred by laminin was assessed. The targets of LY294002, rapamycin, and U0126 (PI3K, mTOR, and MEK, respectively) are shown in the pathway diagram (Fig. 3, left). H69 cells plated on laminin were less susceptible to serum withdrawal–induced apoptosis than those plated on plastic. The addition of a PI3K inhibitor, LY294002, to H69 cells plated on laminin reversed the protective effect of laminin and returned levels of apoptosis to that observed on plastic alone (Fig. 3, top left). Treatment with an mTOR inhibitor, rapamycin, also attenuated the protective effect of laminin; however, not to the same extent as was observed with LY294002 (Fig. 3, top right). The combination of LY294002 and rapamycin abrogated the protection conferred by laminin and increased the level of apoptosis to above what was seen in cells plated on plastic (Fig. 3, bottom left). In contrast, the MEK inhibitor, U0126, not only failed to attenuate the protective effect of laminin but also increased the protective effects of laminin (Fig. 3, bottom right). The basis for this protection is unclear. Taken together, these results show that inhibition of the PI3K/Akt/mTOR pathway but not the MEK/ERK pathway is capable of reversing the protective effect of laminin on SCLC cells.

Because laminin protected SCLC cells from serum withdrawal–induced cell death in a manner dependent upon the PI3K/Akt/mTOR pathway, we sought to explore whether this mechanism

![Figure 3](Figure 3. Biochemical inhibition of the PI3K/Akt/mTOR pathway abrogates laminin-mediated protection of SCLC cells from serum withdrawal–induced apoptosis. The inhibitors used in these studies and their respective targets (left). H69 cells were plated on either plastic (P) or laminin (L) and cultured for 3 days under low-serum conditions followed by the addition of LY294002 (LY, 10 μM/L), rapamycin (R, 10 ng/mL), LY + R (10 μM/L/10 ng/mL), or U0126 (U, 10 μM/L) and cultured for an additional 3 days. Apoptosis was assessed by flow cytometry. *, P < 0.05.)
could underlie resistance to the targeted agent, imatinib mesylate. Imatinib is a small-molecule kinase inhibitor whose known targets include c-Kit, PDGFRs, and bcr-Abl, and imatinib has been approved for use in chronic myelogenous leukemia and gastrointestinal stromal tumor patients (19). In spite of preclinical evidence that c-Kit and its ligand, SCF, are expressed and biologically active in SCLC cells, imatinib has been ineffective in clinical trials of SCLC patients (20). We assessed whether c-Kit, PDGFRα, and PDGFRβ are expressed in the SCLC cell lines used in our studies (Fig. 4A). c-Kit was expressed in all SCLC cell lines, with the lowest levels observed in the H69 cells and the highest levels observed in the H209 and H526 cells. In contrast, PDGFRα or PDGFRβ was not expressed in any SCLC cell line tested but was expressed in NIH3T3 cells, which are known to express PDGFRα and PDGFRβ.

To determine if c-Kit or PDGFRs were coupled to the PI3K/Akt/mTOR or MEK/ERK pathways in SCLC cells, we treated H69 or H209 cells with SCF or PDGF and measured activation of Akt and ERK. SCF but not PDGF increased phosphorylation of Akt and ERK in both cell lines (Fig. 4B). This indicates that c-Kit but not PDGFR is functional and coupled to these signaling pathways in these cells. To confirm that SCF-induced phosphorylation of Akt and ERK was dependent on c-Kit activity, we used imatinib as a c-Kit kinase inhibitor in the presence of SCF. As observed previously, SCF increased phosphorylation of Akt, ERK, and c-Kit in H69, H209, and H526 cells, but pretreatment with imatinib inhibited stimulation by SCF, suggesting that SCF-induced activation of downstream targets was c-Kit dependent (Fig. 4C).

The relationship between laminin-induced activation of signaling pathways and apoptotic response to imatinib was examined next. H69 cells that are plated on plastic undergo apoptosis when exposed to imatinib (data not shown; ref. 21). However, when H69 cells were plated on laminin, the addition of imatinib did not induce apoptosis (Fig. 5A). In fact, imatinib decreased serum starvation induced apoptosis, although this did not reach statistical significance in all experiments. When LY294002 was added to cells plated on laminin, apoptosis increased, and when LY294002 and imatinib were added in combination, cells were sensitized to imatinib-mediated apoptosis (Fig. 5A, top left). Similar results were observed with rapamycin alone or LY294002 and rapamycin in combination (top right and bottom left, respectively). Apoptosis increased with these inhibitors without imatinib. In the presence of imatinib, additional increases in apoptosis were observed. If the decreased levels of apoptosis observed with imatinib alone are taken into account, combining LY294002, rapamycin, or LY294002 and rapamycin with imatinib resulted in

Figure 4. Coupling of c-Kit but not PDGFRs to Akt and ERK in SCLC cell lines. A, six SCLC cell lines were grown in low-serum condition overnight and harvested to examine the expression levels of c-Kit, PDGFRα, or PDGFRβ in immunoblotting experiments. Staining membranes with fast green to show equal loading. NIH3T3 cell lysates were used as a positive control for PDGFRα and PDGFRβ expression. B, H69 and H209 cells were plated in low-serum medium overnight and stimulated with 50 ng/mL SCF or 100 ng/mL PDGF-AB for 10 minutes. The cells were harvested on ice, and lysates were subjected to immunoblotting analysis to measure levels of activated and total Akt and ERK. H157 cells were used as a positive control. C, H69, H209, and H526 cells were plated in low-serum medium overnight and were treated with SCF or imatinib alone, or were pretreated with imatinib (10 μM) for 30 minutes before stimulation with SCF (50 ng/mL) for 10 minutes. Cells were processed as in (B).
greater than additive increases in apoptosis of SCLC cells. The MEK inhibitor U0126 or imatinib alone decreased serum deprivation–induced apoptosis of laminin-adherent SCLC cells. When U0126 and imatinib were combined, the absolute level of apoptosis remained below that observed with untreated laminin-adherent cells (Fig. 5A, bottom left). Thus, inhibition of the PI3K/Akt/mTOR pathway but not the MEK/ERK pathway abrogates laminin-mediated protection of SCLC cells and increases responsiveness to imatinib.

To confirm that the pathway inhibitors were hitting their intended targets in SCLC cells plated on laminin, immunoblotting was done under similar conditions as in Fig. 5A to assess activation of components in each pathway (Fig. 5B). Imatinib alone inhibited Akt activation slightly but did not affect ERK activation. LY294002 inhibited Akt and S6K activation without affecting ERK activation. In contrast, U0126 inhibited ERK activation but not Akt or S6K. Rapamycin alone inhibited S6K phosphorylation but not Akt phosphorylation, which is consistent with inhibition of mTOR, a kinase that lies downstream of Akt and upstream of S6K. Combining imatinib with these signaling inhibitors did not further inhibit the intended signaling pathway. These data show that the induction of apoptosis by combining imatinib with inhibitors of the PI3K/Akt/mTOR pathway is associated with inhibition of the intended targets (PI3K or mTOR). Moreover, the lack of increased
apoptosis when imatinib was combined with U0126 was not due to the fact that U0126 was unable to inhibit MEK.

To extend these results, we treated H209 and H526 cells that were plated on laminin with imatinib and/or LY294002 or U0126 (Fig. 5C). Both cell lines adherent to laminin were resistant to imatinib. LY294002 alone increased apoptosis in H209 cells but not H526 cells. Nevertheless, combining LY294002 with imatinib significantly increased apoptosis in both SCLC cell lines. U0126 alone was protective in H209 cells and had no effect in H526 cells, and the combination of imatinib plus U0126 yielded similar results to that observed with U0126 alone in each cell line. These data support our results with H69 cells in that inhibition of the PI3K/Akt/mTOR pathway but not MEK/ERK pathway sensitizes SCLC cells to imatinib.

To further establish the role of the PI3K/Akt/mTOR pathway in mediating protection of SCLC cells caused by adhesion to laminin, SCLC cells plated on laminin were infected with Ad-dnAkt or Ad-βGal, and apoptosis was assessed in the absence or presence of imatinib under low-serum conditions (Fig. 5D). Similar to results observed with LY294002, adenoviral-mediated overexpression of dominant-negative Akt in laminin-adherent H69 cells increased apoptosis. Combining imatinib with Ad-dnAkt resulted in greater than additive increases in apoptosis. These results support the data using small-molecule kinase inhibitors and suggest that inhibition of the PI3K/Akt/mTOR pathway at multiple points can attenuate laminin-mediated protection and induce sensitivity to imatinib.

**Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not mitogen-activated protein kinase/extracellular signal-regulated kinase pathway increases responsiveness of laminin-adherent small cell lung cancer cells to traditional chemotherapies.** To determine whether laminin-mediated activation of the PI3K/Akt/mTOR pathway could confer resistance to other therapies used in SCLC, we treated H69 and H209 cells plated on laminin with cisplatin or etoposide in the absence or presence of LY294002 or U0126 (Fig. 6). In each cell line, LY294002 significantly increased apoptosis induced by cisplatin or etoposide. Interestingly, U0126 significantly reduced apoptosis induced by cisplatin but did not significantly change apoptosis induced by etoposide. These findings are consistent with our data with imatinib and indicate that the PI3K/Akt/mTOR pathway plays a generalized role in laminin-mediated drug resistance in SCLC cells. Moreover, they suggest that combining inhibition of the PI3K/Akt/mTOR pathway with chemotherapy or targeted agents such as imatinib may overcome resistance conferred by ECM components such as laminin.

**Discussion**

Our study highlights the importance of the PI3K/Akt/mTOR pathway in promoting ECM-dependent SCLC cellular survival and resistance to imatinib or chemotherapy. The first indication that this pathway might be important in SCLC came from Moore et al. who showed that PI3K was constitutively active in SCLC cells and that this promoted anchorage-independent proliferation (22). Multiple mechanisms can contribute to activation of the PI3K/Akt/mTOR pathway in SCLC cells including mutations in the tumor suppressor PTEN (23), expression of specific PI3K isoforms (24), secretion and activity of cytokines or growth factors (13), and adhesion to ECM (14). Because our study is the first to link adhesion to a specific ECM protein, laminin, with activation of the PI3K/Akt/mTOR pathway and resistance to imatinib, these findings may have clinical implications. Targeting the PI3K/Akt/mTOR pathway alone, or with other therapeutic

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**Figure 6.** LY294002 but not U0126 increases the chemotherapy-induced apoptosis in SCLC cells plated on laminin. H69 and H209 cells were plated on laminin in low-serum conditions overnight and treated or not with cisplatin (P, 10 μmol/L) or etoposide (VP, 10 μmol/L), in the absence or presence of LY294002 (LY), U0126 (U), P + LY, P + U, VP + LY, or VP + U for 48 hours. Doses of inhibitors were identical to those used in earlier experiments. Apoptosis was assessed by flow cytometry and P values are shown.
Adhesion to the ECM profoundly affects the behavior of SCLC cells in vitro and in vivo. We showed that the adhesion of H69, H345, H510, and H526 cells to the ECM protein laminin causes dramatic alterations in cell morphology concomitant with increased cell survival in the absence of serum. In addition, we showed that this adhesion is likely mediated by β1-containing integrin receptors. The change in cell shape we observed is characteristic of the reacquisition of an adherent, epithelial-type phenotype that has been described in histologically variant tumor cells within SCLC (25, 26). These cells are highly prevalent in sites of SCLC metastasis (27). Sethi et al. have previously reported that adhesion-mediated alterations in SCLC cell morphology are dependent on integrin activity (4), but our data show that adhesion to laminin confers resistance to serum withdrawal–induced cell death. This might be relevant because before the recruitment of a vascular supply, tumor cells at secondary sites must be able to thrive under growth factor–deficient conditions (7), suggesting that the adhesion-mediated survival we observe may be important for the establishment of SCLC metastasis. In vivo, SCLC cells are surrounded by a dense meshwork of ECM proteins and the interaction of SCLC cell lines with ECM enhances invasiveness (3, 4). Moreover, increased expression of β1 integrin is a poor prognostic factor for patients with SCLC (28).

How does adhesion to ECM and development of an adherent phenotype promote SCLC cell survival in the absence of growth factors? We showed that in H69, H345, H209, and H526 cells, adhesion to laminin increased activation of two serine/threonine protein kinases, Akt and ERK, which paralleled the morphologic change and protection from serum withdrawal–induced cell death. Biochemical or genetic inhibition of the PI3K/Akt/mTOR pathway reversed the protection conferred by laminin. In contrast, inhibition of the ERK pathway had no effect on the protective effects of laminin. Adhesion-mediated activation of Akt and ERK in H69 cells has been previously reported (14); however, increased signaling was in response to nonspecific adhesion not to a defined ECM component such as laminin. In addition, Sethi et al. showed that H69 β1 integrin–dependent adhesion to the ECM protein fibronectin resulted in activation of protein tyrosine kinases (4), presumably due to integrin-mediated activation of receptor tyrosine kinases (RTKs; ref. 29). The PI3K inhibitor LY294002 has also been shown to abolish SCF/SDFα-induced Akt activity in H69 cells and thereby inhibit cellular adhesion (30). Together, these data suggest that the promotion of SCLC cellular survival and morphologic change by ECM relies on activation of the PI3K/Akt pathway.

ECM-mediated activation of the PI3K/Akt/mTOR pathway in SCLC cells promotes resistance to standard and novel therapeutic agents. Traditional agents have included doxorubicin, etoposide, and cisplatin, as well as radiation (4, 13, 14, 31, 32). Our study extends these observations and shows that SCLC cells adherent to laminin are resistant to traditional chemotherapies such as cisplatin and etoposide, as well as to the novel tyrosine kinase inhibitor imatinib.

Although imatinib is approved for the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors (33, 34), its activity in SCLC is limited. Imatinib inhibits the growth of SCLC cell lines in vitro (21, 35), but it has been ineffective at inhibiting tumor growth in preclinical models of human SCLC (36) as well as in a phase II SCLC clinical trial (20). A diagram linking adhesion to laminin, activation of c-Kit, activation of the PI3K/Akt/mTOR pathway, and the apoptotic response to imatinib is shown in Fig. 7. Imatinib has at least three targets, two of which are the RTKs, PDGFRs, and c-Kit. Coexpression of c-Kit and its ligand, SCF, has been shown in ~70% of SCLC tumor specimens and several SCLC cell lines, including H69 cells (30, 37, 38). Surprisingly, the prognostic significance of c-Kit expression in SCLC patients is unsettled (39–41) perhaps because various methods have been used to measure c-Kit, or because the importance of c-Kit depends on the status of and interaction with other molecules such as components of the ECM or growth factor receptors. Expression of the other known target RTK for imatinib, PDGFRs, in SCLC cells was not observed in our study but may have greater importance in stromal cells. Moreover, other relevant unknown targets may exist for imatinib because imatinib can inhibit the growth of SCLC cells in vitro that do not express c-Kit or PDGFR (42).

Together, our data support the hypothesis that enhanced survival of SCLC cells conferred by adhesion to ECM is dependent upon activation of the PI3K/Akt/mTOR pathway and that activation of this pathway is a predictor of resistance to imatinib. Future studies will determine whether combining inhibition of the PI3K/Akt/mTOR pathway with imatinib or other agents is effective in SCLC patients.

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