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Inhibition of Tumor Growth and Angiogenesis by a Lysophosphatidic Acid Antagonist in a Engineered Three- dimensional Lung Cancer Xenograft Model

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Abstract

BACKGROUND—We developed an engineered three-dimensional (3-D) tumor xenograft model of non-small cell lung cancer (NSCLC) in nude mice, and used this model to evaluate a dual-activity inhibitor of lysophosphatidic acid (LPA) biosynthesis and receptor activation.

METHODS—First, BrP-LPA, a pan-antagonist for four LPA receptors and inhibitor of the lysophospholipase D activity of autotaxin, was examined for inhibition of cell migration and cell invasion by human NSCLC A549 cells. Second, A549 cells were encapsulated in 3-D in three semi-synthetic ECMs based on chemically-modified glycosaminoglycans, and injected subcutaneously in nude mice. Tumor volume and vascularity were determined as a function of sECM composition. Third, engineered NSCLC xenografts were formed from A549 cells in either Extracel-HP or Matrigel, and mice were treated with four intraperitoneal injections of 3 mg/kg of BrP-LPA.

RESULTS—First, BrP-LPA inhibited cell migration and invasiveness of A549 cells *in vitro*. Second, tumor growth and microvessel formation for 3-D encapsulated A549 cells *in vivo* in nude mice increased in the order: buffer only < Extracel < Extracel-HP < Extracel-HP containing growth factors plus laminin. Third, tumor volumes increased rapidly in both Matrigel and Extracel-HP encapsulated A549 cells, and tumor growth was markedly inhibited by BrP-LPA treatment. Finally, tumor vascularization was dramatically reduced in the A549 tumors treated with BrP-LPA.

CONCLUSIONS—Engineered A549 lung tumors can be created by 3-D encapsulation in an ECM substitute with user controlled composition. The engineered tumors regress and lose vascularity in response to a dual activity inhibitor of the LPA signaling pathway.

Keywords

LPA antagonist; autotaxin inhibitor; lysophospholipid signaling; engineered tumor xenograft; anti-angiogenesis; hyaluronic acid; Extracel; injectable hydrogel

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INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States,^{1, 2} and among lung cancer patients more than 80% have non-small cell lung cancer (NSCLC). Surgical resection remains the mainstay of therapy for early-stage non-small cell lung cancer and provides the best opportunity for cure.³ However, many of the patients who present with lung tumors are poor surgical candidates due to limited pulmonary function and severe medical comorbidities.⁴ Therefore, chemotherapy is the primary first-line treatment option for the 70–80% of patients who present with locally advanced or metastatic disease.⁵ Although many new anti-angiogenic and anti-metastatic treatments are in preclinical development, a predictive and reproducible animal model of lung cancer that could reliably translate preclinical results to efficacy in human patients remains an unmet need. There is general agreement that tumor xenograft models are useful, but are in need of improvement.^{6–8}

Human lung cancer cells do not grow readily in athymic mice, even when a large number of cells ($> 1 \times 10^7$ cells per mouse)^{9, 10} and/or immunosuppressive agents are used to obtain tumors.¹⁰ Delivery of tumor cells in Matrigel, a three-dimensional (3-D) extracellular matrix (ECM) extracted from murine sarcoma tissue, overcomes some of these shortcomings by providing a growth-enhancing environment that mimics the tumor microenvironment.¹¹ An alternative approach was the use of Extracel, an *in situ*-injectable mimic of the ECM developed for 3-D cell culture and for tissue engineering, to produce cell-seeded semi-synthetic ECMs (sECMs) suitable for drug evaluation.^{12, 13} Indeed, this approach of “tumor engineering” succeeded in (a) increasing the incidence of cancer formation and reducing variability in tumor size, (b) enhancing growth of organ-specific cancers with good tumor-tissue integration, (c) improving vascularization and reducing necrosis in the tumor, (d) reducing cancer seeding on adjacent tissues or organs, and (e) improving the overall health of the animals.¹⁴ Low-take ovarian and colon cancer cell lines, breast cancer cells, and metastatic pancreatic cancer cells formed vascularized, localized, orthotopic tumors in nude mice.^{14, 15} Recently, this tumor engineering model has been validated for evaluation of new anti-cancer compounds that modify signal transduction pathways essential for tumor cell migration and proliferation.¹⁶

The *in situ* crosslinkable sECM Extracel is fully chemically defined and non-immunogenic, and its composition, compliance, and even rate of crosslinking can be customized for specific cell types for *in vitro* and *in vivo* applications.¹² The critical importance of angiogenesis in growth and metastasis of lung cancers has led to investigation of an increasing number of antiangiogenesis agents for all types of pulmonary malignancies.¹⁷ This angiogenesis is mediated by factors such as vascular endothelial growth factor (VEGF).¹⁸ Immobilization of a thiol-modified heparin derivative in the sECM provided a component that mimicked the heparan sulfate proteoglycans (HSPGs) of native ECMs.¹⁹ This HSPG-mimetic sECM allowed spatiotemporal control of the delivery of single or dual growth factors, including bFGF, VEGF, angiopoietin-1, and KGF,²⁰ and elicited a formation of mature vasculature *in vivo*.^{21–23} Growth-factor loaded Extracel-HP has also been shown to recruit endogenous mesenchymal stem cells *in vitro*.²⁴

Lysophosphatidic acid (LPA) is a bioactive lipid that promotes cancer cell proliferation, migration and survival through activation of cell surface G protein-coupled receptors.^{25–27} In particular, the ascites of ovarian cancers is rich in LPA and has been implicated in growth and invasion of ovarian cancer.²⁸ Recently, LPA was found to stimulate hypoxia-inducible factor-1-independent VEGF expression to promote tumor angiogenesis through activation of the c-Myc and Sp-1 transcription factors.²⁹ Moreover, LPA reduced the cellular abundance of the tumor suppressor p53 in A549 lung carcinoma cells, which express endogenous LPA receptors.³⁰

Inhibiting the production of LPA is also a relevant therapeutic target.^{31, 32} The lysophospholipase D (lysoPLD) activity of the tumor-associated protein autotaxin (ATX) converts lysophosphatidylcholine (LPC) to LPA.^{33, 34} ATX, one of the forty most upregulated genes in invasive cancers, is implicated in cell motility and tumor invasion, metastasis, and neovascularization.^{35, 36} Thus, simultaneously abrogating signaling through the GPCRs and attenuating LPA production by ATX suggested dual activity metabolically-stabilized LPA analogs,^{37, 38} such as those found to reduce breast cancer in orthotopic xenograft models,¹⁶ would also have potential as therapeutic agents for treatment of lung cancer.

We describe herein the optimization of a new lung cancer xenograft model using human A549 NSCLC cells encapsulated in a 3-D sECM. First, we modified the composition of the modular sECM by addition of growth factors and laminin peptides to improve tumor growth characteristics. Next, we compared A549 tumors produced in the optimized Extracel-HP formulation to those generated in Matrigel to evaluate the ability of BrP-LPA, a dual activity LPA receptor antagonist and autotaxin inhibitor (Figure 1, Table 1), to induce tumor regression and reduce tumor vascularization. The sECM model demonstrated that a biologically relevant context for A549 cells could be customized, with full user control over composition of the matrix, to evaluate new therapeutic agents for lung cancer *in vivo*.

MATERIALS AND METHODS

Crosslinkable sECM hydrogel

The injectable sECMs tested included Extracel-XX (a 2% w/v hydrogel) and Extracel-HP (Glycosan BioSystems, Salt Lake City, UT). Hydrogels generally formed within 20 min following the mixing of the components according to the manufacturer's instructions. For this study, Extracel-HP hydrogels were formed by adding Heprasil into Gelin-S and PEGDA (v:v:v=1:1:0.5). For hydrogels containing growth factors, VEGF and bFGF were added into the mixture before gelation to give final concentrations of 600 ng/mL VEGF and 600 ng/mL bFGF. For the hydrogel containing the laminin peptide, stock synthetic peptide CGIKVAVGY ("L4") was added to the Extracel-XX or Extracel-HPG prior to adding the crosslinker PEGDA to the thiol-modified macromonomer mixture ³⁹ to give a final concentration of 2 mg/mL.

Cells

A549 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured using the recommended complete medium, F-12K medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 U/mL penicillin (GIBCO), 100 µg/mL streptomycin (GIBCO).

Scratch wound assay

A549 cells were plated in triplicate into six-well plates at a concentration of 3×10^5 cells per well. Approximately 48 h later, the confluent cell layers were carefully scratched using sterile pipette tips. Nonadherent cells and cellular debris were removed by washing with PBS. For the first set of experiments, fresh medium alone (with 0.5% FBS) or medium with 0.5% FBS containing 10 µM or 40 µM of BrP-LPA^{16, 37} was added into the wounded monolayers. For the second set of experiments, medium was supplemented with 1 µM 20:0 LPA (Echelon Biosciences, Inc., Salt Lake City) or with 1 µM 20:0 LPA plus 10 µM or 40 µM of BrP-LPA. Cells were observed under the microscope and digitally photographed at different times. Inhibition of migration was assessed when the wound in the control was closed and quantified by using ImageJ.⁴⁰

Invasion assay

The invasive behavior of cells was determined *in vitro* using 24-well transwell inserts fitted with 8 μ m pore size PET membranes, which were coated with 0.368 mg/mL Matrigel.⁴¹ A suspension of cells (100 μ L of 5×10^4 cells/mL) in serum-free medium with or without 10 μ M BrP-LPA was added to triplicate inserts, and 600 μ L medium supplemented with serum was used as the chemoattractant in the lower chamber. After 24 h, the cells that did not invade through the pores were removed, and cells that passed through the filter on the underside of the membrane were stained with the Diff-Quick Staining Set (IMEB Inc., San Marcos, California) and counted. Ten fields of cells were counted for each well, and the mean number of cells per field was calculated. Each experiment was performed in triplicate and repeated at least twice.

Lung cancer xenograft optimization

Female 4–6 week old *nu/nu* mice (Charles River Laboratories, Wilmington, MA) were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) as approved by the University of Utah Institutional Animal Care and Use Committee (IACUC). Before inoculation, A549 cells were trypsinized and resuspended in different Extracel compositions (Glycosan BioSystems, Inc. Salt Lake City, UT) with a final concentration of 5×10^7 cells/mL, and the resulting suspension was mixed gently by vortexing. The following ECM compositions were examined with six mice per group: Extracel-XX, Extracel-HP alone, and Extracel-HP containing 600 ng/mL bFGF and 600 ng/mL VEGF as well as 2 mg/mL of the L4 peptide. For the negative controls, cells were injected in PBS only. For each composition, a 200 μ L aliquot of the cell suspension was injected subcutaneously into two injection sides on the dorsum of each mouse.

Lung cancer xenograft treatment models

Treatment with BrP-LPA, synthesized and provided by Dr. Honglu Zhang (U of Utah), was performed as previously described¹⁶ with modifications. The mice were randomly divided into two treatment groups and two control groups (six mice per group). In the first controlled experiment, a suspension of A549 cells in Extracel-HP with added L-4 (2 mg/mL) but without growth factors (5×10^7 cells/mL) was prepared, and prior to gelation, a 200- μ L aliquot was injected subcutaneously into the dorsum of each of the twelve mice in the control and treatment groups. In the second controlled experiment, a suspension of A549 cells was prepared in Matrigel (5×10^7 cells/mL) at 4 °C, and a 200 μ L aliquot was injected subcutaneously into the dorsum of each of the twelve mice in the control and treatment groups. For all groups, tumors were allowed to form for 2.5 weeks. Then, each treatment group received i.p. injections of BrP-LPA (3 mg/kg) twice per week for 2 weeks; the control groups were injected i.p. with physiological saline.

The tumor sizes were measured with digital calipers and calculated as follows: tumor size (mm^3) = [width (mm)]² $\times$ [length (mm)]/2. The anti-tumor effects of the treatment were evaluated in terms of the lowest T/C values (%), where T is the relative mean tumor size of the treatment group, and C is the relative mean tumor size of the control group at any given time. The animals' body weights were measured at the time of tumor measurement to monitor for any drug toxicity. Mice were sacrificed after 7 weeks, and the tumor tissues were removed for histology and processed for H&E staining and immunohistochemistry using an anti-CD31 antibody as previously described.¹⁶

Statistical Methods

Data from *in vitro* and *in vivo* experiments were processed to express as the mean $\pm$ SD of at least triplicate determinations. Statistical comparisons were performed by Student's *t* test, and differences were considered significant at $p < 0.05$.

RESULTS

In vitro inhibition of cell migration

The effects of BrP-LPA without or with LPA stimulation on the cell migration of A549 lung cancer cells were tested using a scratch wound assay (Figure 2). A549 lung carcinoma cells express endogenous LPA receptors LPA₁₋₃, 30 and LPA₁ is the most important GPCR mediating cell migration of normal and neoplastic cells.⁴² In the first set of experiments, after treatment with different concentrations of BrP-LPA, cells were allowed to migrate into the denuded area for 0, 8 and 16 h. By 16 h, untreated control cells completely filled the scratched area, while the migration of A549 cells was significantly decreased ($p < 0.05$) by 40 μ M BrP-LPA when compared to untreated cells. In a second set of experiments, cell migration was stimulation with 1 μ M arachidoyl (20:0) LPA, and the ability of BrP-LPA to antagonize this activation was examined. Importantly, even under these stringent conditions, 40 μ M BrP-LPA significantly decrease cell migration compared to the LPA-stimulated cells. Inhibition of cell migration at both time points suggests that these effects are not due to inhibition cell of proliferation.

In vitro invasion assay

To examine the ability of BrP-LPA to inhibit the invasiveness of A549 cells, an *in vitro* invasion assay using a modified Boyden chamber was employed. A549 lung cancer cells showed prominent invasion through Matrigel-coated transwell membranes (Figure 3a). Treatment with BrP-LPA inhibited the invasion ($p < 0.05$) for A549 cells (Figure 3b), suggesting that BrP-LPA has the potential to reduce the metastatic potential of lung cancer cells *in vivo*.

Lung cancer xenograft optimization

Subcutaneous injection of A549 cells suspended in PBS only, Extracel-XX, Extracel-HP, and Extracel-HP plus growth factors and L4 into *nu/nu* mice resulted in tumor growth at all sites of injection (Figure 4). As anticipated, the slowest rate of tumor growth occurred in the buffer-only injected cells, while cells in the 2% w/w Extracel-XX, in analogy to the previous tumor engineering studies with breast, ovarian, and colon cancer, generated significantly larger tumors 769 ± 251 mm³ over the 7-week time course. Even without added GFs, Extracel-HP alone provided better tumor growth environment than Extracel-XX, reaching 949 ± 148 mm³. This demonstrates that the addition of the HSPG-mimetic crosslinkable heparin component alone could improve tumor growth. The addition of the VEGF + bFGF combination, and the covalent addition of the IKVAV-containing laminin peptide appeared to provide only a modest improvement (1012 ± 322 mm³) over the simpler Extracel-HP.

H&E staining allowed visualization of the cellular organization within the tumor mass, and revealed that the tumors were mainly composed of carcinoma cells in the untreated groups with blood vessels. Immunohistochemistry using anti-CD31 antibodies identified the microvascular pattern within the NSCLC tumors (Figure 5a). Further calculation of the microvessels density (MVD) was performed when stained CD31 immunohistochemical tests were obtained. The results indicated that sECMs with immobilized heparin modulated GF release and promoted the development of functional microvessel network (Figure 5b).

Tumor regression in engineered NSCLC tumor xenografts

Two hypotheses were tested simultaneously with the *in vivo* models. First, we asked whether Extracel-HP could be used in place of the commonly-used Engelbreth-Holm-Swarm mouse sarcoma-derived Matrigel for 3-D encapsulation for rapid growth of vascularized tumor tissue. The analytical data accompanying Matrigel indicate that the predominant growth factors present include TGF- β , EGF, IGF, bFGF, and PDGF. Second, we tested whether the engineered tumors in each ECM substitute would be susceptible to treatment with BrP-LPA, which had been shown to cause breast tumor regression in an engineered orthotopic xenograft model.¹⁶

Subcutaneous injection of A549 cells suspended in either Extracel-HP (Figure 6) or Matrigel (Figure 7) formed tumors *in vivo*, which in the untreated control group after 7 weeks grew to $864 \pm 211 \text{ mm}^3$ in Extracel-HP and $1058 \pm 427 \text{ mm}^3$ in Matrigel. It is not surprising that A549 cells suspended in Extracel-HP + L4 (Figure 6a) had a slightly lower growth rate than cells encapsulated in Matrigel (Figure 7a), since the tumor-derived Matrigel has the full spectrum of sarcoma-derived growth factors and other basement membrane components which provide cancer cells with a naturally favorable microenvironment. What is remarkable is how small the difference is. With only a minimal laminin peptide, without growth factors, and without a myriad of tumor ECM factors, a simple crosslinked hydrogel composed of a network of chemically-modified HA, gelatin, and heparin recapitulated the majority of the cues provided by the tumor microenvironment. Moreover, Extracel was readily handled at room temperature rather than at 4 °C.

After 2 weeks of tumor growth, mice in the treatment groups received intraperitoneal injections of BrP-LPA (3 mg/kg), twice per week for 2 weeks. The dose was selected on the basis of the previous dose of this pure *anti* diastereoisomer of BrP-LPA that was found to be effective in reducing tumor volume and vascularity in an orthotopic breast cancer model.¹⁶ In both the Extracel (Figure 6a) and Matrigel (Figure 7a) groups, a significant reduction of tumor size was observed by the end of the first week of treatment when compared to the respective control groups. During treatment, estimated tumor mass did not exceed 10% of body weight, and the exposure to BrP-LPA for 7 weeks did not reduce the body weight significantly (data not shown), indicating that the compound was well tolerated with minimal off-target toxicity. We had previously observed a similar lack of general toxicity in a 10 mg/kg dose of the mixed diastereoisomers of BrP-LPA.¹⁶

After completion of the 2-week treatment course, tumor sizes in both treatment groups were significantly decreased (Figure 6a, Figure 7a). More importantly, tumors continued to regress for an additional three weeks following the final BrP-LPA injection in both the Matrigel and Extracel-HP matrices. The figures show statistically significant differences at each time point, from midway through the drug doses through to the end of the experiment.

At 7 weeks, animals were sacrificed, and the tumors were surgically removed and prepared for histology analysis. The tumor samples in the BrP-LPA treatment groups were significantly smaller than the tumor tissues in the control group (Figure 6b, Figure 7b). H&E staining revealed an irregular arrangement of tumor cells and an increased number of blood vessels (Figure 6c, Figure 7c) in the untreated controls compared with in the BrP-LPA treated group (Figure 6d, Figure 7d). An endothelial layer covering tumor vasculature was observed using immunohistochemical staining with anti-CD31 antibody (Figure 6e, Figure 7e), which was reduced in both treatment groups (Figure 6f, Figure 7f). Finally, the quantification in six different fields of three separate slides for each treatment group (Figure 6g, Figure 7g) showed highly significant reduction of angiogenesis ($p < 0.05$).

DISCUSSION

We tested two hypotheses using engineered NSCLC tumor xenografts. First, we asked whether a simple, modular sECM such as Extracel could be used in place of the mouse sarcoma-derived Matrigel to support rapid growth of vascularized tumors. Second, we examined how engineered tumors in each ECM substitute would respond to systemic treatment with BrP-LPA, an LPA antagonist/ATX inhibitor that had been shown to cause breast tumor regression in an engineered orthotopic xenograft model.¹⁶ These two issues are discussed separately below.

Improved xenograft models

Current animal models of lung cancer have low rates of tumor growth and metastasis.⁴³ Growing human lung cancer cells in athymic mice has been problematic,^{9, 10} even when immunosuppressive agents and a high density of tumor cells are used to obtain tumors.¹⁰ In the basement membrane, metastatic lung tumor cells attach preferentially to laminin.^{44, 45} Laminin stimulates tumor cell adhesion,⁴⁶ collagenase IV production,⁴⁷ cell motility,^{48, 49} and the formation of metastasis.⁴⁵ One potential opportunity to improve xenograft quality would thus be to include laminin or a laminin derived peptide to increase the tumorigenicity of 3-D encapsulated lung cancer cells *in vivo*.

Cell-matrix interactions in 3-D are an essential part of the microenvironment of all cells, including tumor cells.⁵⁰ The tumor-derived ECM product Matrigel has seen widespread use in distinguishing gene expression and phenotypic differences in cells growth in 3-D⁵¹ modeling tissue-specific signaling and organ function in 3-D⁵², and in modeling glandular epithelial cancers in three-dimensional cultures.⁵³ The use of Matrigel as a 3-D cell delivery system rich in tumor-derived growth factors greatly increases the incidence of cancer formation *in vivo*.^{54–56}

Despite these advantages, there are procedural disadvantages in the use of Matrigel. For example, the syringe and needle need to be pre-cooled at -80°C , and all procedures -- from re-suspending cells to preparing the cells for injection -- need to be carried out at 4°C in a very short time to avoid premature gelation and thus clogging of the needle. These problems are exacerbated during orthotopic injections in surgical models, resulting in increased experimental variability and failed injections. In addition, batch-to-batch variability and lack of user control of the composition of the 3-D matrix further complicate the use of Matrigel. The sECM Extracel addresses many of these drawbacks, offering a room-temperature-injectable 3-D hydrogel vehicle for cell delivery.^{12, 14} Moreover, in the absence of GFs, the pro-angiogenic effects of the oligosaccharide degradation products of HA have been implicated in the growth of non-necrotic, vascularized tissues from normal¹³ and tumor-derived cells.¹⁴

In order to identify a sECM based on Extracel that would be optimal for NSCLC cells, we investigated the encapsulation of A549 cells in three different Extracel compositions. Since epithelial cells on stiffer substrates have higher moduli and exhibit a more stretched and organized actin cytoskeleton than those on softer substrates,⁵⁷ we examined the 2% (w/v) hydrogel Extracel-XX, which is stiffer than the 1% (w/v) hydrogel Extracel-X. We also tested Extracel-HP, a sECM that contains an immobilized derivative of heparin, thereby mimicking an HSPG-like microenvironment for presentation and sequestration of basic growth factors (GFs). Finally, we noted that metastatic tumor cells attach preferentially to laminin, the predominant glycoprotein in basement membranes.^{44, 45} Laminin stimulates tumor cell adhesion, collagenase IV production, cell motility, and the formation of metastasis. The final composition tested was a modification of Extracel-HP containing a covalently-linked IKVAV peptide⁵⁸ plus VEGF and bFGF growth factors. Surprisingly, the

addition of the growth factors and the laminin peptide only showed a modest improvement over just Extracel-HP itself in terms of neovascularization and microvessel formation in the engineered lung cancer xenograft tumors.

Therapeutic potential of ATX inhibitors and LPA antagonists

LPA GPCRs and ATX present two promising, druggable, yet under-exploited targets for cancer therapy.^{30–31} 59 ATX, a potent motogen in metastatic cancers³⁵ produces a continuous output of LPA,^{33–34} potentially abrogating the action of an LPA antagonist. By blocking ATX, the autocrine/paracrine loop that involves the ATX-mediated production of LPA is reduced.^{32–39} Although ATX is product feedback inhibited,⁶⁰ using LPA to inhibit ATX would be counterproductive. Thus, analogues of LPA that retained ATX inhibitory activity yet did not activate LPA GPCRs are needed. BrP-LPA is a potent pan-LPA GPCR antagonist and ATX inhibitor,¹⁶ inhibits cell migration, invasion, and proliferation, and thus has clear therapeutic potential.⁶¹ In contrast, analogues of cyclophosphatidic acid (ccPA) were selective inhibitors of ATX but lacked agonist activity for LPA_{1,2,3}.⁶¹ These ccPA analogues inhibited cancer cell invasion *in vitro* and suppressed metastasis of melanoma cells *in vivo*. Reduction of melanoma cell migration *in vitro* was also demonstrated for several small-molecule ATX inhibitors.⁶² However, ATX inhibition alone is insufficient, since it is necessary to suppress receptor activation by endogenous LPA as well as reduce LPA production for maximal therapeutic efficacy.

Conclusions

We have described a useful new engineered lung cancer xenograft model using human A549 NSCLC cells encapsulated in Extracel, a 3-D crosslinked network of chemically-modified gelatin and glycosaminoglycans. The addition of the growth factors VEGF and bFGF, and the addition of a laminin peptide only modestly improved tumor growth. Growth of A549 NSCLC tumor xenografts in Extracel-HP *in vivo* were compared with NSCLC xenografts in Matrigel, and the models were used to evaluate the ability of BrP-LPA, a dual activity LPA receptor antagonist and autotaxin inhibitor, to induce tumor regression and reduce tumor vascularization. The sECM model demonstrated that Extracel-HP provides a useful new material for A549 xenografts, and allows for complete experimental control over composition of the matrix during the *in vivo* evaluation of new therapeutic agents for the treatment of lung cancer.

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Figure 1.
Chemical structures of LPA and dual action LPA receptor antagonist-autotaxin inhibitor.

Figure 2.

Effects of BrP-LPA on A549 NSCLC cell migration. In one set of experiments, a confluent A549 cell monolayer was scratched with a pipette tip and then treated with BrP-LPA (10 or 40 μ M) and compared with untreated cells (CTL). To demonstrate antagonism in the presence of stimulation, 20:0 LPA (1 μ M) was mixed with BrP-LPA (10 or 40 μ M) and compared with activation by 1 μ M of 20:0 LPA without antagonist at 8 h and 16 h. Cell migration at 0, 8 h and 16 h post-injury was assessed photographically (panel **a**). Data for BrP-LPA with or without LPA induction were quantified using Image J (panel **b**). Asterisks indicate significant differences from controls (CTL or LPA only) at $p < 0.001$ (*).

Figure 3. Effects of BrP-LPA on invasion of A549 NSCLC cells through Matrigel-coated membranes. Panel **a**: representative fields of A549 NSCLC cells that invaded using cell suspension in serum-free medium with (BrP-LPA) or without 10 μ M BrP-LPA (CTL). Panel **b**: mean value of invading A549 cells (*, $p < 0.05$).

Figure 4.

Effect of matrix on A549 NSCLC tumor growth. Cells were suspended in Extracel-HP (5×10^7 cells/mL), Extracel-HP with growth factors (600 ng/mL bFGF, 600 ng/mL VEGF and 2 mg/mL L4), Extracel-XX, and PBS. In each group, cells were injected subcutaneously (s.c.) on the back of *nu/nu* mice (200 μ L) with two injection sites per mouse.

Figure 5. Photographs of representative tumor sections from animals in each group. Hematoxylin and eosin (H&E) staining (Panel **a**). Masson's trichrome staining of extracellular matrix (blue) in the tumor tissue sections and hematoxylin counterstained. Microvessels were stained with anti-PECAM antibody (CD31) and hematoxylin counterstained. Brown staining indicates positive staining for endothelial cells. Panel **b**: Microvessel density (MVD) in the hydrogel plugs were visualized, and determined by counting six vascularized fields in each section (* indicates $p < 0.05$; ** indicates $p < 0.01$).

Figure 6. Treatment model using Extracel-HP encapsulated cells. A549 NSCLC cells were suspended in Extracel-HP (5×10^7 cells/mL) containing L4 peptide but no growth factors and injected subcutaneously on the back of *nu/nu* mice (200 μ L). Treatments (3 mg/kg BrP-LPA, i.p.,) are marked with arrows. Panel **a**: Time course of tumor growth. Panel **b**: Comparison of tumor samples excised from untreated control group (upper) and treatment group (lower). H&E staining of control tumors (panel **c**) and treated tumors (panel **d**). Immunohistochemical staining with CD31 specific endothelial markers for control group (panel **e**) and treatment group (panel **f**) showing relative angiogenesis within the cancer cells as blood vessels per area (panel **g**) (*, $p < 0.05$).

Figure 7. Treatment model using Matrigel encapsulated cells. A549 NSCLC cells were suspended in Matrigel (5×10^7 cells/mL) and injected subcutaneously on the back of *nu/nu* mice (200 μ L). Treatments (3 mg/kg BrP-LPA, i.p.,) are marked with arrows. Panel **a**: Time course of tumor growth. Panel **b**: Comparison of tumor samples excised from control (upper) and treatment groups (lower) (*, $p < 0.05$). Immunohistochemical staining with CD31 specific endothelial markers for control (panel **e**) and treatment groups (panel **f**) show relative angiogenesis within the cancer cells by the measurement of blood vessels per area (panel **g**).

Table 1
Summary of biological activities of LPA and BrP-LPA on LPA GPCRs (modified from Ref. 16).

	LPA ₁	LPA ₂	LPA ₃	LPA ₄	LPA ₅
16:0 LPA	Agonist	Agonist	Agonist	Agonist	Agonist
<i>anti</i> isomer of 16:0 BrP-LPA (Fig.1)	Antagonist	Antagonist	Antagonist	Antagonist	Antagonist
<i>syn</i> isomer of 16:0 BrP-LPA (not shown)	Antagonist	Antagonist	Antagonist	Antagonist	Partial agonist