The Serum Glycoprotein Fetuin-A Promotes Lewis Lung Carcinoma Tumorigenesis via Adhesive-Dependent and Adhesive-Independent Mechanisms

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Abstract

Fetuin-A is a serum glycoprotein in the cystatin family associated with the regulation of soft tissue calcification. We tested the role of systemic fetuin in tumor cell growth and metastasis by injecting Lewis lung carcinoma (LLC) cells into fetuin-A null and their wild-type (WT) littermate control C57BL/6 mice via the tail vein, s.c., and intrasplenic routes. In the experimental metastasis assay, the lungs of the WT mice were filled with metastatic nodules, whereas the lungs of the fetuin-A null mutant mice were virtually free of colonies at the end of 2 weeks. Lung colonization responded to the levels of serum fetuin-A in a dose-dependent manner, as observed by the formation of half as many colonies in mice heterozygous for the fetuin-A locus compared with homozygous WT mice and restoration of lung colonization by the administration of purified fetuin-A to fetuin-A-null mice. Serum fetuin-A also influenced the growth of LLC cells injected s.c.: fetuin-A-null mice developed small s.c. tumors only after a substantial delay. Similarly, intrasplenic injection of LLC cells resulted in rapid colonization of the liver with metastasis to the lungs within 2 weeks in the WT but not fetuin-A null mice. To examine the mechanism by which fetuin-A influences LLC colonization and growth, we showed that LLC tumor cells adhere to fetuin-A in a Ca^{2+}-dependent fashion, resulting in growth of the tumor cells. These studies support the role of fetuin-A as a major growth promoter in serum that can influence tumor establishment and growth. (Cancer Res 2005; 65(2): 499-506)

Introduction

Fetuin-A is the bovine homologue of the human α 2HS glycoprotein (Ahsg). The fetuin-A family encompasses a related group of glycoproteins synthesized by the liver and have been designated fetuin in sheep, pig and cow, 63-kDa phosphoprotein in rat, and countertrypin in mouse (1). The serum concentration of the bovine protein is high during fetal development ∼20 mg/mL but drops to a level of ∼0.60 mg/mL in the adult serum (2). The fetal levels in human (∼1.5 mg/mL) is lower than in bovine and yet adult levels of Ahsg in the blood of humans is comparable to the bovine blood levels at ∼0.50 mg/mL. Ahsg/fetuin-A is a member of the cystatin family of proteins and is a negative acute phase protein (1, 3, 4) in that its serum concentration drops after trauma, infection, and certain malignancies. Recently, a second protein of the fetuin family called fetuin-B was described in humans and rodents (1). Based on domain homology, overall conservation of cysteine residues and chromosomal assignments of the corresponding genes in these species, fetuin-B was described as a paralogue of fetuin-A.

Fetuin-A has been linked to a number of cellular functions such as brain development (2), bone remodeling, and immune function (5, 6). It can also mimic and antagonize the anti-mitogenic effects of transforming growth factor-β in test tube assays, in cell culture, and in intact animals (7, 8). Some of these functions however, can be attributed to a number of proteins, which copurify with fetuin-As, and has therefore made it difficult to assign a particular function to fetuin-A without ambiguity (9). Fetuin-A deficiency in mice is associated with a mild soft tissue calcification phenotype (5). The ectopic calcification becomes dramatically exacerbated when the fetuin-A deficiency is combined with the genetic background DBA/2, which is sensitive to dystrophic calcification (10). These data prove that fetuin-A is a potent systemic inhibitor of unwanted ectopic calcification.

Fetuin-A, as the major constituent of bovine fetal serum, has long been suspected of being the active cell growth promoter in serum. However, this assignment was challenged on the grounds that fetuin-A is a sticky molecule and thus the contaminating growth factors that copurify with it are most likely responsible for the growth stimulation (9). The availability of fetuin-A null mice has enabled us to revisit this problem in the present studies. We have examined the role of fetuin-A in the colonization of lungs and livers of fetuin-A null C57BL/6 mice and their wild-type (WT) littermate controls by Lewis lung carcinoma (LLC) cells following tail vein and intrasplenic injections. We have also examined s.c. growth of LLC cells in the fetuin-A null, heterozygous, and WT littermate controls. We report that the lack of fetuin-A in these mice significantly protects them from developing tumors *in vivo*. Furthermore, fetuin-A is capable of promoting the growth of more aggressive tumor cells but not benign and normal cells *in vitro*. The cells adhere and divide on immobilized fetuin-A in the presence of 2 mmol/L Ca^{2+} ions. The binding of fetuin-A to the surface of the cells triggers the PI3 kinase/Akt signaling pathway and cell proliferation.

Materials and Methods

Animals. The strategies for producing fetuin-A null mutant mice were as described (5). The original null mutant strain 129,B6,$Ahsg^{tm,Mbl}$ was crossed to pure-bred C57BL/6 mice from a commercial breeder (Charles River, Sulzfeld, Germany) for five successive generations resulting in the strain B6,$Ahsg^{tm1Wja}$ according to Institute for Laboratory Animal Research

nomenclature. Animals used in this study originated from sibling crosses of heterozygous offspring of the fifth back-cross (>99.9% pure C57BL/6 genetic background). Littermate homozygous B6,*Ahsg*$^{-/-}$ and B6,*Ahsg*$^{+/+}$ mice were used. All animal experiments conformed to established procedures and were approved by the local animal experimentation review board.

For genotyping during mouse breeding, DNA was obtained by proteinase K digestion and extraction of tail biopsies taken at the time of weaning as described. The identity of animals was confirmed by PCR analysis. WT–specific primers were Fet1: 5′-GAGACTGTGACTTCCACATCC-3′ and Fet2: 5′-GGTTCCATTATTCTGTGTGTTG-3′, whereas the null mutant (knockout) primers were Neo1: 5′-ATCTCCTGTCATCTCACCTTGC-3′ and Neo2: 5′-ACCCCACCCCCACCCCCGTAGC-3′. The PCR conditions were optimized at 95°C for 15 minutes for 1 cycle and then 94°C; 1 minute and 57°C; 1 minute and 72°C; 1 minute for 4 cycles and lastly 91°C; 30 seconds and 57°C; 30 seconds and 72°C; 30 seconds for 28 cycles. The product (25 μL) was resolved on a 1.5% agarose gel. The WT product is a 500-bp band, whereas a knockout product is 650 bp.

Cell Culture. LLC is a spontaneously occurring lung cancer in C57BL/6 mice. The tumor cell line was obtained from American Type Culture Collection (Rockville, MD) and is routinely maintained in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin. Other cells used in the study were breast epithelial cell lines, BT-549, galectin-3 transfected subclone of BT-549 named 11-9-1-4 (11–13), MDA-MB-231, and ZR-75-1. These were likewise cultured in the DMEM/F12 supplemented with serum as described above. The DMEM/F12 medium (Sigma, St. Louis, MO) used in this study does not contain Ca^{2+} and Mg^{2+} ions. When supplemented with 10% FBS, all the divalent ions are derived from the serum. Concentrations of Ca^{2+} and Mg^{2+} ions in the serum-free medium were adjusted accordingly.

Experimental Metastasis Protocol. The LLC cells were injected via the tail vein of WT and fetuin-A null mutant C57BL/6 mice. The cells were injected at 300,000 cells per mouse in a total volume of 100 μL. The mice were observed daily for any sign of respiratory distress and were euthanized by CO_2 after 2 weeks. The lungs were removed and inflated with Bouin's fixative. Livers were also examined.

Preinjection of Mice with Purified Fetuin-A. Fetuin-A was purified from 4 mL of blood from sacrificed mice. The blood was collected by orbital bleeds in heparin tubes, centrifuged, and plasma obtained. The plasma was subjected to ammonium sulfate precipitation (2 mol/L), followed by centrifugation and the precipitate dissolved in 50 mmol/L sodium phosphate buffer containing 0.1 mol/L NaCl (pH 7.4). Aliquots of this were loaded on wheat germ agglutinin-agarose column and recycled 10 times at a constant flow rate of 0.8 mL/min at 22°C. After extensive washing with the phosphate buffer, the bound fraction was eluted with the same buffer containing 0.2 mol/L GlcNAc. The fetuin-A fraction was concentrated and dialyzed against phosphate buffer and finally purified by gel filtration (Sephacryl 200), concentrated, and aliquoted. To monitor the clearance of fetuin-A from the blood of null mice following injection, 300 μg of mouse fetuin-A were injected i.v. and blood (100 μL) drawn by orbital bleed. Blood collected in heparinized tubes was centrifuged to obtain plasma. Purified bovine fetuin-A was purchased from Sigma.

Western Blot for Fetuin-A Levels. Western blot analysis for plasma fetuin-A levels was done as described previously (5), using rabbit anti-bovine fetuin-A antibody (Chemicon, Temecula, CA). This antibody cross-reacts with mouse fetuin-A.

Intrasplenic Injection Protocol and S.c. Growth of LLC Cells. LLC cells (300,000 cells per animal) in 100 μL PBS were injected into the spleens of anesthetized WT and fetuin-A null C57BL/6 mice. The spleens were removed after allowing a few minutes for the cells to travel into the livers. All the animals recovered well after the surgery and their health status was monitored daily. After 15 days, most of the animals showed signs of discomfort and were immediately sacrificed by CO_2 asphyxiation. The organs were fixed in formalin and prepared for histopathology.

The LLC cells were also injected s.c. in the dorsal region of fetuin-A null and WT littermate controls. The cells were injected at 300,000 cells per mouse in a total volume of 100 μL of PBS. The mice were observed daily and

tumor growth monitored and measured for up to 7 weeks. The length and width (in mm) of the tumors were measured by Verner Caliper and tumor volume determined as 0.5 × width^2 × length.

Adhesion of Cells to Fetuin-A–Coated Wells. Cells (4×10^4 cells per well) were allowed to adhere to microtiter wells coated with 100 μL of 0.5% fetuin-A or asialofetuin-A in serum-free medium in the absence and presence of Ca^{2+} ions. After 1 to 2 hours of incubation, the nonadherent cells were washed off using the same medium and the adherent cells fixed with 5% glutaraldehyde in PBS followed by staining with crystal violet and photographed. To the stained cells in the microtiter wells, 100 μL of 0.1 mol/L acetic acid were added per well and the absorbance of the eluted stain was recorded at 570 nm using a microplate reader MRX (Dynex Technologies).

Growth and Analysis of Cell Cycle of LLC in the Presence and Absence of Fetuin-A. To the wells of a 96-well tissue culture microtiter plate, 2×10^4 cells per well were added in DMEM/F12 (lacking Ca^{2+} and Mg^{2+} ions) containing 0.5% bovine fetuin-A (w/v) and 0.04 or 2 mmol/L $CaCl_2$. As positive control, cells were also allowed to grow in DMEM/F12 supplemented with 10% FBS (complete medium). Growth was measured every 24 hours for at least 5 days. The number of viable cells was determined by the Alamar blue assay (14, 15). In this assay reducing conditions, which reflect mitochondrial activity turns the Alamar blue dye from blue to pink with increased fluorescence at 590 nm after excitation at 544 nm. Every 24 hours, Alamar blue dye was added to the conditioned medium in the wells of microtiter plates at a dilution of 1:20 and initial fluorescence taken using a luminescence spectrophotometer LS 55 (Perkin-Elmer, Norwalk, CT) with a fluorescence plate reader attachment. The cells were allowed to incubate at 37°C for 1 hour after which another reading was taken. This process was repeated at least twice and the average of the increase in fluorescence per hour in arbitrary units of fluorescence was determined for each experimental condition.

LLC cells were also allowed to interact with fetuin-A in suspension (anchorage independent) in the absence and presence of Ca^{2+} ions. The cells were allowed to grow in suspension in Eppendorf tubes at 37°C for at least 3 days and the viable cells determined daily by the Alamar blue assay as described above.

The transition of the LLC cells through the cell cycle was analyzed by Guava PCA following the manufacturers protocol. Briefly, the LLC cells were cultured until 70% confluent, followed by serum starvation for 24 hours. The serum-free medium in one flask (experimental) was replaced with serum-free medium containing 0.25% w/v of fetuin-A and 2 mmol/L Ca^{2+}. After another 24-hour period, the cells in both flasks were harvested by 2 mmol/L EDTA, pelleted at $450 \times g$ for 5 minutes, and the supernatant discarded. The cells were resuspended in 1 mL PBS, pelleted once more, and supernatant aspirated leaving a small amount of PBS (~ 100 μL) at the bottom of the centrifuge tube. The cells were resuspended in the residual PBS and 1 mL of ice cold ethanol added dropwise per 1×10^6 cells. The fixed cells were refrigerated for 12 hours, pelleted, and 1 mL of staining solution (25 μg/mL propidium iodide, 0.1% triton-X100, and 0.2 mg/mL DNase-free RNase in PBS) added. The cells were incubated for 30 minutes at room temperature in the dark. The samples were then transferred to 1.5-mL microcentrifuge tubes for data acquisition.

Modulation of the Adhesion and Growth of Tumor Cells by Pharmacologic Inhibitors of PI3 Kinase and Akt. Mounting evidence suggests that the PI3 kinase/Akt signaling pathway is an essential growth signaling pathway particularly for tumor cells (16). The determination of the effects of various growth factors and drugs on the PI3 kinase/Akt pathway in culture cells often necessitates the use of serum-free medium. We therefore hypothesized that fetuin-A in the serum is responsible for the background activity of the PI3 kinase/Akt signaling. The cells were cultured in complete medium until 75% confluent, after which they were detached with EDTA, resuspended in serum-free medium and allowed to adhere to fetuin-A coated (500 μg per well) wells of 96-well microtiter plate in serum-free medium. The adhesion was in the presence of 2 mmol/L Ca^{2+} and graded doses of LY294002 (0-200 μmol/L). After 1 hour, the nonadherent cells were washed off and the number of adherent cells determined by fluoresceine diacetate (17) and expressed as percentage of cells in control wells (no drug). The LLC cells (2×10^4 cells per well) were also grown in serum-free medium containing 0.25% of fetuin-A and

2 mmol/L Ca^{2+} in 96-well microtiter plate, in the absence (control) and presence of graded doses (0-200 μmol/L) of the specific inhibitors for PI3 kinase and Akt. The cells were allowed to grow for 4 days after which the old medium was replaced with new and the number of live cells determined by the Alamar blue assay and expressed as percentage of cells in the untreated control wells.

Phosphorylation of Akt in the Absence and Presence of PI3 Kinase Inhibitors. The cells were cultured in T75 culture flasks until 70% confluent at which point the cells were replated in serum-free medium containing 2 mmol/L calcium. Some flasks contained either 50 nmol/L of wortmannin or 50 μmol/L of LY 294002 in the serum-free medium. After 24 hours of serum starvation, fetuin-A (0-0.5% w/v) was added to the flasks. The cells were allowed to incubate for three more hours, harvested by 2 mmol/L EDTA, lysed in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7), 50 mmol/L NaF, 1% Brij 35, 0.5% Triton X-100, 2 mmol/L sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] and protein concentration of the lysate determined. The lysate was mixed with sample buffer and resolved in 10% PAGE. After Western transfer, the membrane was probed with antibodies to phospho-Akt473, phospho-Akt308, and total Akt (Signal transduction), with acid stripping after each antibody. The Western protocol, as stipulated by the supplier of the antibodies, was followed. The experiment was repeated but instead of replating cells in culture flasks after serum deprivation, the cells were incubated without and with graded doses of fetuin-A in microcentrifuge tubes for 3 hours at 37°C, lysed and analyzed as above.

Results

Colonization of Lungs by LLC Cells. WT C57BL/6 (B6,*Ahsg*$^{+/+}$) and fetuin-A null mutant (B6,*Ahsg*$^{-/-}$) litter mates (Fig. 1*A*) were injected via the tail veil with syngeneic LLC cells. Within 2 weeks of injection, the WT mice were visibly riddled with metastatic tumor nodules compared with the lungs of the fetuin-A null mice (Fig. 1*B* and *C*). When present, the nodules in the lungs of fetuin-A–deficient mice were much smaller than those appearing in the lungs of the WT mice (data not shown). The experiment was repeated with quantitatively similar results (Fig. 1*D*). There were no metastatic nodules in the livers of either fetuin-A WT or null mutant mice. This experiment indicated that fetuin-A contributes to the colonization of the lung by LLC tumor cells.

The levels of serum fetuin-A influenced lung colonization in a dose-dependent manner. Tail vein injection of LLC cells into mice heterozygous for the *Fetuin-A* locus resulted in an intermediate number of lung nodules after 3 weeks (Fig. 2*A*). Fetuin-A null mice were then injected i.v. with either purified mouse or bovine fetuin-A, and the levels of circulating fetuin-A measured by Western blot analysis. Purified fetuin-A injected into the null mice was detectable for up to 6 days (Fig. 2*B*); thus, animals were reinjected each week. The levels of serum bovine fetuin-A at 7 days postinjection were similar to that in the heterozygous mice (Fig. 2*C*). Preinjection of either purified murine or bovine fetuin-A resulted in an increase in lung nodules in the experimental metastasis assay to levels intermediate between the WT and null mice, and similar to that in the heterozygous mice (Fig. 2*A*). Thus, serum fetuin-A supports the development of lung nodules in a dose-dependent manner.

Growth of LLC Cells in the Livers of Mice. To determine if the effect of fetuin-A on tumor colonization was specific for the lung, we injected LLC cells into the livers of both WT and fetuin-A null mice via the spleen. After only 15 days of growth, the livers of WT were grossly enlarged and heavier compared with those of fetuin-A null mice. The histopathology of the livers of WT mice represented by Fig. 3*A* (*left*), showed significant tumor proliferation with extensive

coalescing areas of tumor cells with atypical nuclei, numerous mitotic cells and central areas of necrosis and hemorrhage. In contrast, fetuin-A null mice livers showed less aggressive tumor growth with mild multifocal areas of tumor infiltration randomly scattered throughout the liver parenchyma (Fig. 3*A*, *right*, *arrowhead*). We also examined the lungs of the spleen-injected mice.

Figure 1. Colonization and growth of LLC cell in the lungs of fetuin-A WT and null mutant mice. *A,* mice were genotyped as WT, null mutant (*KO*) or heterozygous (*HET*). The LLC cells (300,000 per mouse) in a total volume of 100 μL of PBS were injected via the tail vein into two groups (5 animals per group), fetuin-A WT and null mutant C57BL/6 mice. The mice were euthanized after 2 weeks by CO$_2$ asphyxiation, the lungs removed and filled with Bouin's fixative, and photographed (*B*). The number of metastatic nodules on the lungs of fetuin-A WT and null mutant mice were counted (blind study) in two separate experiments (*C* and *D*).

Whereas there were no visible metastatic nodules, the lungs of the WT mice contained aggregates of three to five tumor cells as well as single cells peppered throughout the walls of the alveoli and in capillaries (Fig. 3*B*, *left*), and the tumor cells showed characteristic morphology of LLC (18). The lungs of the fetuin-A null mice on the other hand, were devoid of any micrometastatic foci and there were no significant microscopic lesions (Fig. 3*B*, *right*).

The development of lung and liver nodules in the experimental metastasis assays is dependent on both seeding of the lung and liver with circulating tumor cells and the growth of these cells into visible nodules. To address the possibility that fetuin-A influences the growth of tumor cells, LLC cells were injected s.c. into WT and fetuin-A null mice. By the end of 3 weeks following tumor
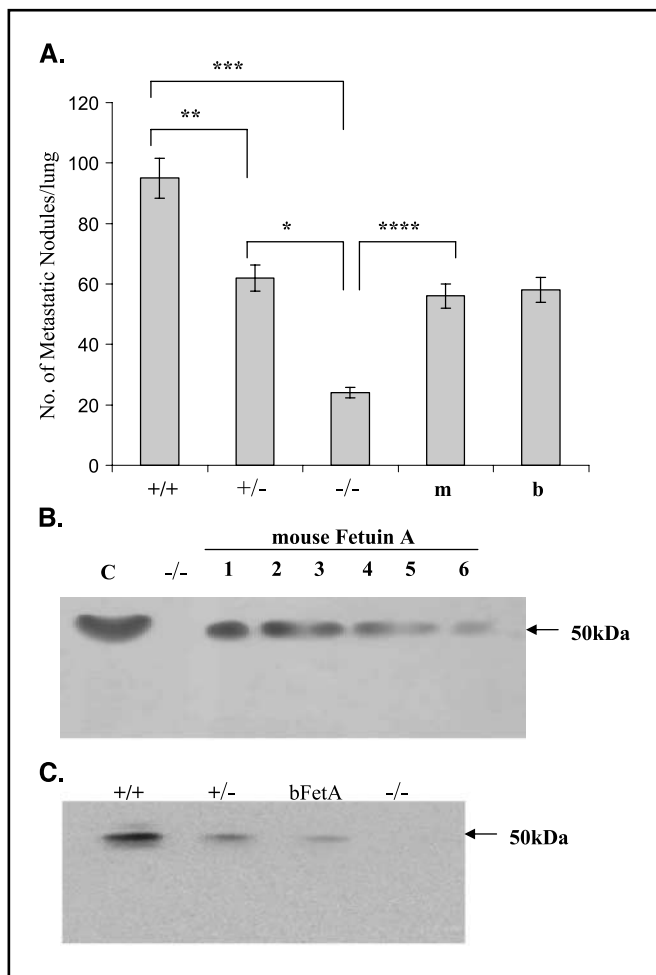
Figure 2. Fetuin-A expression and growth of LLC in the lungs of C57BL/6 mice. *A,* LLC cells (300,000 per mouse) were injected into fetuin-A WT controls (+/+), heterozygous (+/−), null (−/−), null preinjected with purified mouse fetuin-A (*m*), and null preinjected with purified bovine fetuin-A before tumor inoculation (*b*). The mice were euthenized 3 weeks later and number of metastatic nodules per lung determined. *, $P < 0.01$; **, $P < 0.04$; ***, $P < 0.0001$; ****, $P < 0.001$. *B,* clearance rate of purified mouse fetuin-A injected into null mice was determined by Western blot. Fetuin-A null mouse was injected with 300 μg of purified mouse fetuin-A and 5 μg of plasma proteins analyzed after 24 hours (*lane 1*), 48 hours (*lane 2*), 72 hours (*lane 3*), 96 hours (*lane 4*), 120 hours (*lane 5*), and 144 hours (*lane 6*). As controls, 5 μg of plasma proteins from WT mouse (*lane C*) and fetuin-A null mouse (−/−) were also analyzed for fetuin-A expression by Western blot. *C,* 5 μg of plasma proteins from WT mouse (+/+), heterozygous (+/−), fetuin-A null injected with 900 μg of purified bovine fetuin-A, 7 days before blood withdrawal (*bFetA*), and fetuin-A null mouse (−/−) were likewise assayed for fetuin-A.
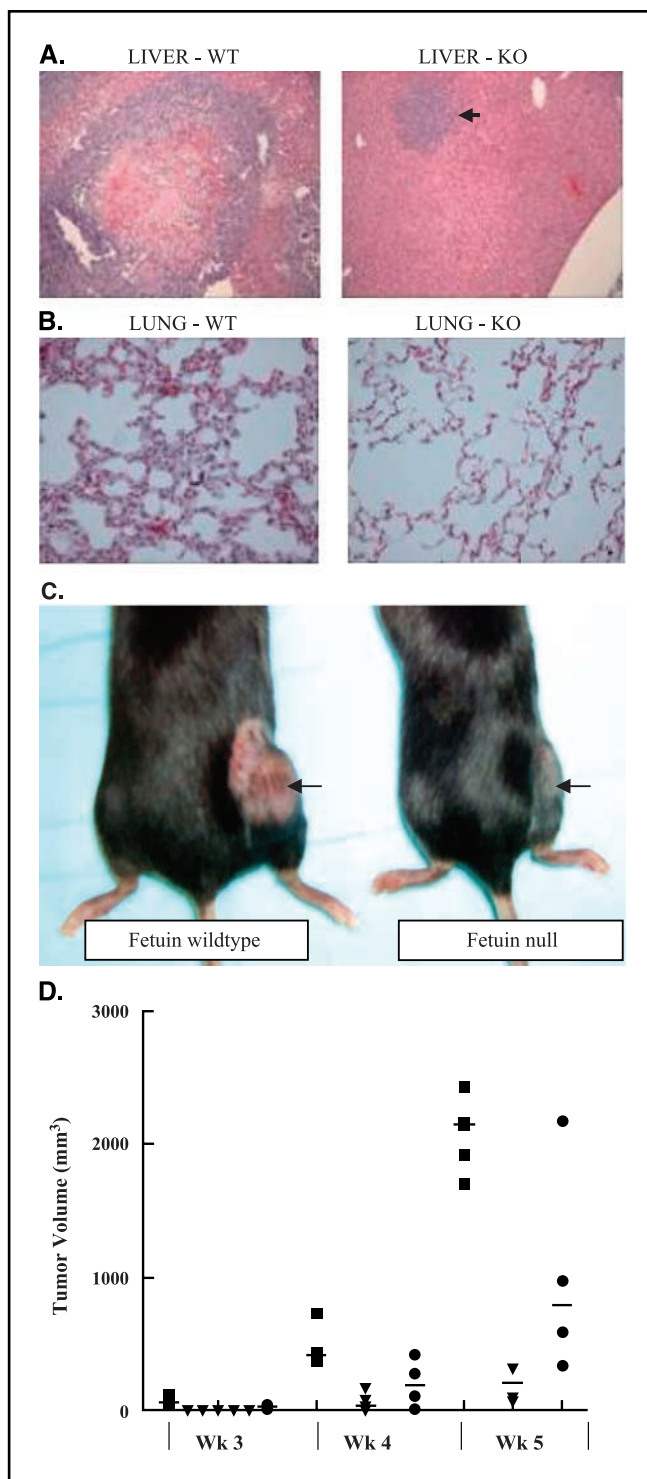
Figure 3. Growth of LLC cells in the liver, lungs, and s.c. tissue of C57BL/6 mice. In *A* and *B*, LLC cells were injected directly into the spleens of anaesthetized animals, and the livers and lungs prepared for histopathology 15 days later. Representative liver section of WT C57BL/6 (*A, WT*) showed increased tumor proliferation with extensive coalescing areas of tumor cells with atypical nuclei, numerous mitotic cells, central areas of necrosis and hemorrhage. Livers of fetuin-A null mice (*A, KO*) on the other hand, showed reduced tumor proliferation with small focal areas of tumor cell aggregates (*arrowhead*). *B,* lungs of WT animals showed microclusters of tumor cells, whereas the lungs of null mice (*KO*) lacked micrometastasis. *C,* s.c. growth of LLC tumor in fetuin-A WT and null mutant mice 4 weeks after the inoculation of the cells. *D,* raw values of tumor volumes in each group. WT (■), fetuin-A null (▼), and fetuin-A null mice preinjected with bovine fetuin-A (●).

inoculation, none of the fetuin-A null mice had visible tumor growth in the skin (Fig. 3C, *right*). In contrast, WT littermate controls as well as the null mice preinjected with fetuin-A had visible tumors by the end of 3 weeks (Fig. 3C, *left*). The tumors grew steadily such that by week 7, zmost of the mice had to be euthanized due to heavy tumor burden. Visible sign of tumor growth was observed in one of the fetuin-A null mice by week 4 (Fig. 3D). The s.c. growth of tumors in the null mice preinjected with purified fetuin-A was higher than growth seen in the null mice but lower than growth in the fetuin-A WT mice (Fig. 3D). Thus, circulating fetuin-A influenced the growth of LLC cells independent of any influence on trapping of circulating tumor cells in the lung and the liver.

Fetuin-A Promotes Adhesion of LLC Cells. The colonization of the lung and liver in the experimental metastasis assays can be influenced by the lodging of circulating LLC cells. Fetuin-A has been suggested to be an extracellular adhesive molecule, but the mechanism by which it mediates cellular adhesion is poorly understood. To determine if LLC cells adhere to fetuin-A, cells were plated on tissue culture dishes coated with fetal bovine fetuin-A or asialofetuin. LLC cells adhered and spread rapidly on fetuin-A but not asialofetuin-A in a Ca^{2+} ion–dependent fashion (Fig. 4A). This type of adhesion represents a novel mechanism, distinct from the one mediated by integrins that require Mn^{2+} or Mg^{2+} ions (19). It should be noted here that breast tumor cells as well as LLC cells also adhere to immobilized fetuin-A in the presence of either Mg^{2+}/ Mn^{2+} ions (data not shown). To determine if tumor cells uniformly adhere to fetuin-A, we tested the adhesion of two breast cancer cell lines. ZR-75-1 breast cancer cells showed minimal adherence to fetuin-A (Fig. 4B and C). In contrast, 11-9-1-4 cells, a galectin-3– transfected subclone of BT-549 that has been shown to be metastatic in nude mice and has strong adhesive interactions with the extracellular matrix adhesive molecules laminin and collagen IV (20, 11), shows substantial adherence to fetuin-A. LLC cells are intermediate between these cells in their ability to adhere to fetuin-A. Interestingly, LLC and 11-9-1-4 cells are considered aggressive and metastatic, whereas ZR-75-1 cells are not, although the direct relationship between these observations and adherence to fetuin-A is not clear.

Fetuin-A Promotes the Growth of LLC Cells. To further investigate the role of fetuin-A in the growth of LLC cells, we questioned whether the fetuin-A–mediated adhesion was sufficient to stimulate growth in the cells. Fetuin-A stimulates the growth of LLC in a dose-dependent manner with optimal concentration being in the region of 0.2% to 0.5% (w/v; data not shown). We incubated these cells in tissue culture microtiter plates in serum-free medium containing 0.5% fetuin-A and calcium. As a control, the cells were also allowed to grow in complete DMEM/F12 medium containing 10% of FBS. Cell number was determined at 27, 48, 96, and 120 hours postplating. Over a 5-day period, the cells grew more rapidly in the presence of 0.5% fetuin-A and 2 mmol/L Ca^{2+} compared with cells growing in complete medium which contains ∼ 0.25% fetuin-A, divalent ions, and other growth and adhesion factors (Fig. 5A). Growth in the presence of fetuin-A and low concentration of Ca^{2+} ions (0.04 mmol/L) was highly reduced. Interestingly, the LLC allowed to grow in suspension in Eppendorf tubes also proliferated in the presence of fetuin-A and Ca^{2+} ions (Fig. 5B). This anchorage-independent growth was highly reduced in the absence of fetuin-A. The experiment was repeated thrice and for longer periods with similar results.

Normally, serum deprivation for at least 24 hours arrests most tumor cells in the G_1 phase of the cell cycle. We therefore

hypothesized that fetuin-A, because of its potential to replace serum in the growth medium, activates cell cycle signaling mechanisms and enables the transition into the S and G_2-M phases. After only 24 hours in fetuin-A, the transition of the cells through the S and G_2-M phases of the cycle were clearly much higher compared with the control cells (Fig. 5C). As expected, majority of the control cells were blocked in the G_1 phase (Fig. 5C).

Fetuin-A/Ca^{2+}–Mediated Signaling in Tumor Cells. The pharmacologic inhibitor of PI3 kinase (LY 294002) acutely inhibited
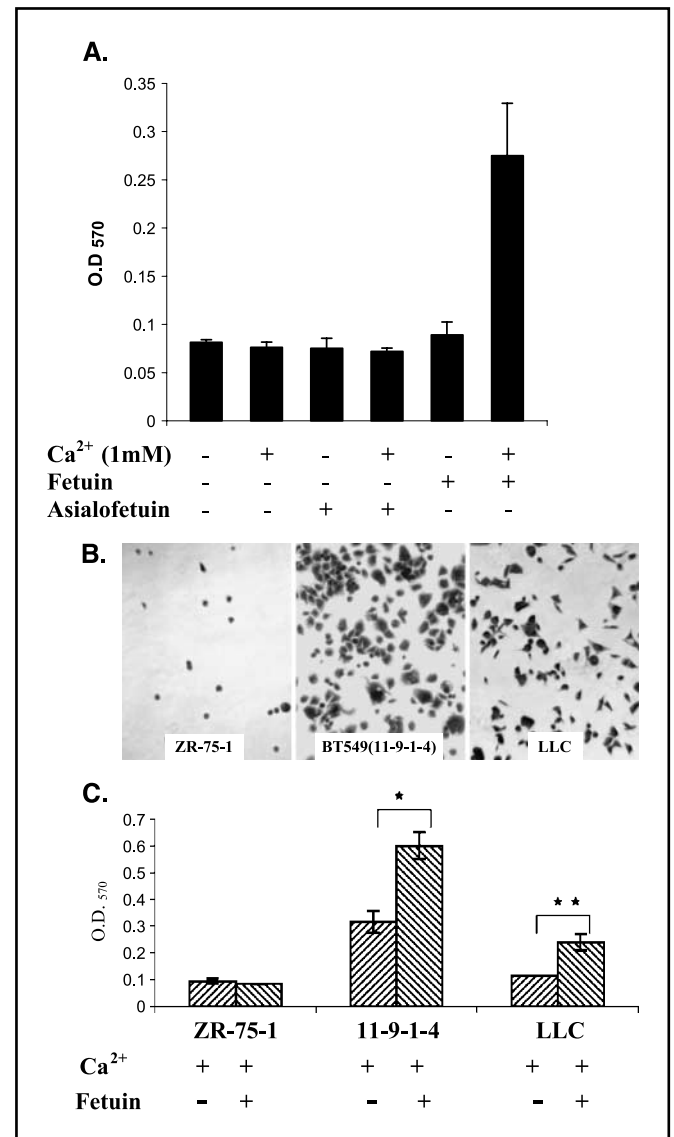
Figure 4. Adhesion of LLC and breast carcinoma cell lines to fetuin-A–coated wells. *A*, wells of 96-well tissue culture plate were coated without or with 0.5% fetuin-A or asialofetuin-A for 1 hour at 37°C and LLC cells added in presence and absence of Ca^{2+} ions as indicated and allowed to adhere for 2 hours at 37°C. Adherent cells were fixed in 5% glutaraldehyde, stained with crystal violet, and the stain eluted with acetic acid and absorbance determined at 570 nm. *B*, wells of a 96-well tissue culture plate were coated without or with 0.5 % fetuin-A as above and breast carcinoma cells, ZR-75-1 and BT549 clone 11-9-1-4 and the LLC added and allowed to incubate for 2 hours at 37°C in serum-free medium containing 2 mmol/L Ca^{2+} ions. The adherent cells were fixed in 5% glutaraldehyde, stained with crystal violet, and photographed. *C*, stain was eluted from quadruplicate wells containing these cell lines and absorbance determined as described above. *, $P < 0.01$; **, $P < 0.005$.

the fetuin-A/Ca^{2+}–mediated adhesion of LLC cells (Fig. 6*A*). More interestingly, after 4 days in culture, the synthetic inhibitor of Akt (IL-6-hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl carbonate) and LY 294002-mediated cell death in LLC cells in the presence of fetuin-A/Ca^{2+} with LC$_{50}$ of ∼ 30 and 9 μmol/L respectively (Fig. 6*B*). To show phosphorylation of Akt upon contact of LLC cells with fetuin-A, both adherent and cells in suspension were analyzed. The direct measurement of phosphorylated forms of Akt by Western blot analysis upon stimulation of LLC cells with fetuin-A after serum deprivation, revealed increased fractions of Akt phosphorylated on Thr-308 and Ser-473 relative to the total Akt protein (Fig. 6*C*). The fraction of total Akt phosphorylated at Thr-308 in control cells (*lane 1*) was estimated to be 0.09. Upon exposure to

0.25% and 0.5% fetuin-A (*lanes 2* and *3*), this fraction jumped to 0.34 and 0.46, respectively, and the level of phosphorylation was not affected much by preincubation with inhibitors of PI3 kinase (*lanes 4* and *5*). The fraction of total Akt phosphorylated at Ser-473 in control cells was 0.10. Upon exposure to 0.25% and 0.5% fetuin-A, the phosphorylated fraction increased to 0.24 and 0.26, respectively. In the presence of 0.5% fetuin-A and 50 nmol/L wortmannin, there was no phosphorylation on Ser-473. In the presence of 0.5% fetuin and 50 μmol/L LY 294002, the phosphorylated fraction was reduced to 0.16. This increased phosphorylation was also evident in nonadherent cells or cells growing in suspension (data not shown). The PI3 kinase inhibitors wortmannin and LY 294002 were more effective in inhibiting the phosphorylation of the Ser-473 residue compared with the Thr-308. The data suggest that fetuin-A/Ca^{2+} supports the growth of LLC cells by promoting antiapoptotic signaling mechanisms that include PI3 kinase/Akt.

Discussion

In the present work, we have analyzed the role(s) that fetuin-A plays in the liver, lung and s.c. growth of LLC cells *in vivo*. The long-standing ambiguity and contentions regarding the possible contribution of fetuin-A in cell growth was the impetus for present studies (9). We have shown that fetuin-A is a critical growth factor not only in the colonization of C57BL/6 livers and lungs by LLC cells following experimental metastasis protocol but also the s.c. growth of these cells.

The prometastatic activity of fetuin-A is in sharp contrast to other studies demonstrating that bovine fetuin-A from fetal blood in the presence of zinc ions, can induce apoptotic cell death and abrogate tumor incidence in nude mice (12). It was postulated in the report that fetuin-A could induce the synthesis of alkaline phosphatase, which has been shown to trigger apoptosis in some cells (21, 22). In our studies, fetuin-A was not saturated with Zn^{2+} ions as was the case reported by Yu and Tsai (12). Indeed, we have recently observed that presence of as little as 2 mmol/L of Zn^{2+} in fetuin-A completely abrogates its capacity to support cellular adhesion and growth of tumor cells (unpublished information).

Whereas experimental metastasis models can be considered artificial in the sense that the malignant cells have bypassed a number of key steps in their forage from the primary tumors, the final steps of extravasation, adhesion to foreign extracellular matrices, and growth in an ectopic site are similar to those arising from spontaneous metastasis (23). Lung colonization is dependent on the lodging of circulating cells in the lung microcirculation and the growth of those cells into visible colonies. We show here that LLC cells adhere to fetuin-A in a calcium-dependent manner. The calcium-mediated adhesion that is distinct from integrin-mediated adhesion (requiring Mg^{2+} or Mn^{2+}) is considered novel. The LLC cells, however, can also adhere to immobilized fetuin in the presence of Mg^{2+} or Mn^{2+} ions (data not shown). This type of adhesion, which is likely to be mediated by integrins, could be due to the contaminating adhesion proteins, which copurify with fetuin-A (2, 9). In breast tumor cell lines, we have observed that the calcium-mediated adhesion was predominant only in metastatic or more aggressive but not in benign or normal breast epithelial cells. This adhesion mechanism also requires the presence of sialic acid residues on fetuin-A as evidenced by the lack of adhesion of LCC cells to asialofetuin. Apart from sialic acid residues, overall glycosylation status of fetuin-A may also influence its cell adhesive properties in the presence of calcium. It has been determined by
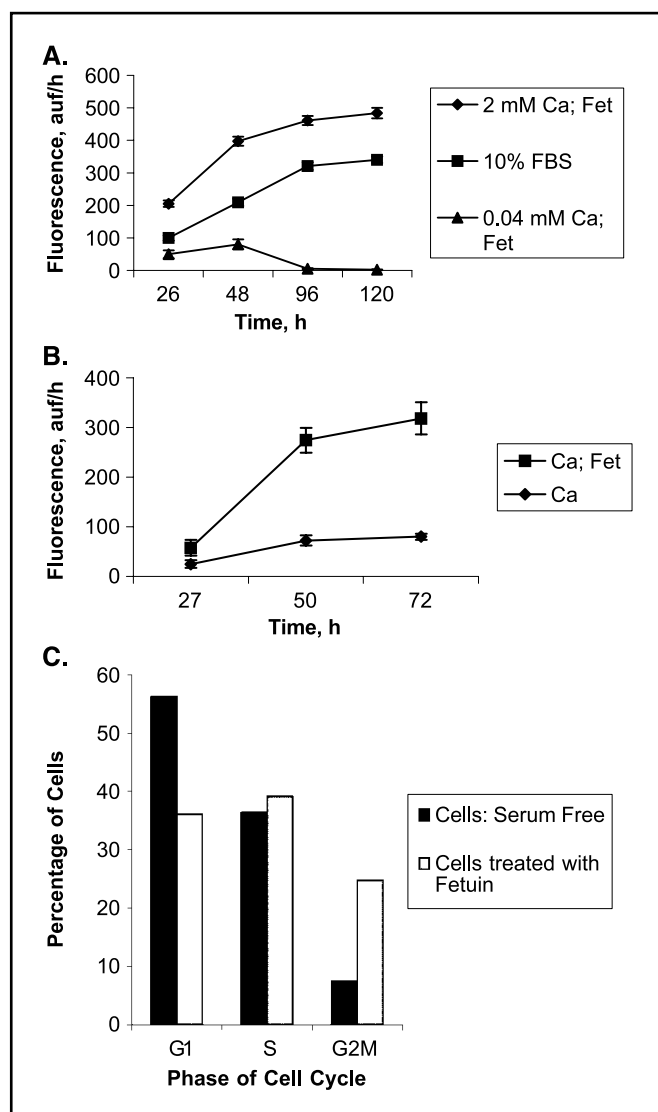
Figure 5. Fetuin-A–mediated growth of LLC cells. *A,* LLC cells (2 × 10^4 cells per well) were plated in 96-well cell culture plates in serum-free DMEM/F12 containing 2 mmol/L Ca^{2+} and 0.5% fetuin-A, 10% FBS, and 0.04 mmol/L Ca^{2+} and 0.5% fetuin-A. *B,* LLC cells (2 × 10^4) were incubated in Eppendorf tubes for various time points at 37°C in a total volume of 700 μL. Cells were allowed to grow in suspension in serum-free DMEM/F12 containing 2 mmol/L Ca^{2+} plus 0.5% fetuin-A (■) and 2 mmol/L Ca^{2+} only (♦). The LLC cells were also serum starved for 24 hours and then exposed to serum-free medium without (*solid bars*) and containing 0.25% fetuin-A (*B*) for another 24 hours. Cell cycle profile was analyzed using PI staining and % cells in the various phases indicated in *C.*

lectin blot assays that whereas bovine fetuin-A and Ahsg from HepG2 cell contain both N- and O-linked sugars, normal human serum Ahsg contains only N-linked sugars (24).

In addition to its role as adhesion promoter, probably the most important activity of fetuin-A, based on the present data, is the *in vitro* and *in vivo* growth signaling. There have been tell-tale signs that some cells, particularly tumor cells, may harbor cell surface receptors for fetuin-A (13, 25). We and others have recently shown that annexins on the cell surface serve as receptors for fetuin-A (17, 26). Whereas the precise physiologic role of annexins is yet to be established, they are emerging as multifunctional proteins, which take part in a variety of key physiologic functions (27–30). They are essential for the adhesion and growth of cells, particularly tumor cells (31). The binding of fetuin-A to the annexins on the cell surface in a calcium-dependent fashion, is most likely the initiator and rate-determining step of the growth signaling suggested by the present report. Our data show and support earlier studies that fetuin-A can replace FBS in growth medium (2, 9), suggesting that it is the active growth factor in FBS. We therefore envision a working model where fetuin-A binds to either annexin-2 or annexin-6 (17) on the cell surface in a calcium-dependent manner, culminating in the PI3 kinase activation. This then leads to phosphorylation of Akt on both its Thr-308 and Ser-473 residues, leading to downstream growth related signaling. This model is supported by our data showing that exposure of cells to fetuin-A after the serum block allows the tumor cells to transition through the cell cycle. This pathway seem to be more active in the later stages of tumor progression because our earlier studies on skin carcinogenesis using fetuin-A null and WT mice did not reveal such dramatic differences between the two groups of mice. The absence of fetuin-A did not alter tumor onset or conversion of benign papillomas to squamous cell carcinomas but reduced the number of tumors per mouse by 30% (32).

The present studies show that fetuin-A supports anchorage-independent growth of LLC cells. This adhesion-independent growth that is a hallmark of transformed cells usually require among other growth factors, FBS in the growth medium. In these studies, we replaced FBS with fetuin-A and Ca^{2+} ions and showed enhanced growth of LLC cells in suspension. More interestingly, the PI3 kinase/Akt signaling pathway which our data suggest is activated by fetuin-A /Ca^{2+} has been established as a major signaling pathway in anchorage-independent growth of tumor cells (33–35). Whereas adhesion-independent growth requires other growth factors and proteins such as galectin-3 (36), the present data propose a central role for fetuin-A in this tumor-specific growth mechanism.

It is evident from the present study that the growth of LLC cells in the lungs, liver, and s.c. tissue depends on the circulating levels of fetuin-A in the blood. It should be noted that an immune rejection phenomenon is not responsible for the reduced growth of tumor cells in the null mice for two reasons: first, littermate controls were used and second, purified fetuin-A could reconstitute the growth of tumor cells in the null mice. We therefore hypothesize that *in vivo*, where anchorage-independent growth plays a big role in the growth of primary tumors as well as metastatic colonies, the presence of fetuin-A in the tumor microenvironment is crucial, and defining all the major players of this novel pathway should lead to new strategies for designing the next generation of anticancer drugs.

Recently, we reported that the mild calcification phenotype of fetuin-A–deficient mice is dramatically exacerbated when the fetuin-A null mutation in mice is combined with the genetic
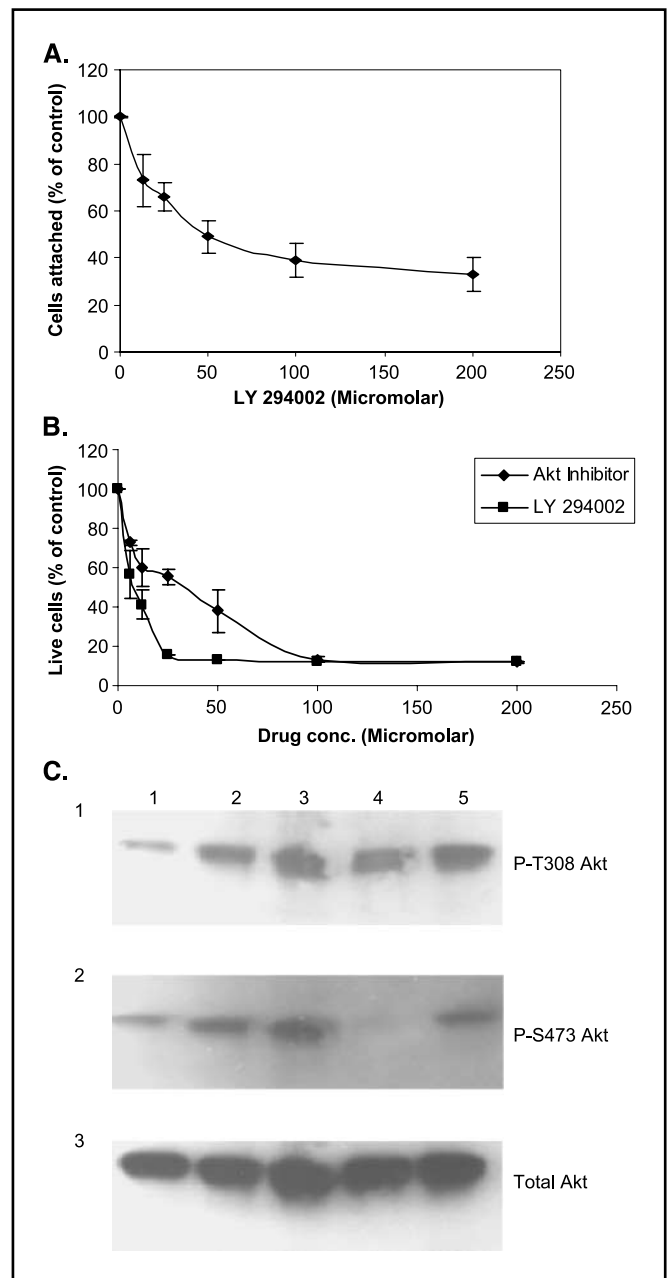
Figure 6. The effect of PI3 K/Akt inhibitors on rapid adhesion and growth of tumor cells. *A,* LLC cells were allowed to adhere to the wells of a 96-well microtiter plates coated with fetuin-A (500 μg per well) in the presence of 2 mmol/L Ca^{2+} and graded doses of LY 294002 (PI3 kinase inhibitor; 0-200 μmol/L). After 1 hour, the adherent cells were determined and expressed as % control (no drug). *B,* LLC cells were grown in serum-free medium containing 0.25% fetuin-A, 2 mmol/L calcium, and graded doses of LY 294002 and Akt inhibitor (0-200 μmol/L). Cells were allowed to grow for 4 days (96 hours) after which, the medium was replaced with fresh serum-free medium containing Ca^{2+} and the number of live cells in the wells determined by the Alamar Blue assay and expressed as % live cells in the untreated control wells. *C,* cells were cultured in complete medium until ∼70% confluency. The cells were then detached and replated in serum-free medium containing 2 mmol/L Ca^{2+} without (*lanes 1-3*) or with 50 nmol/L wortmannin (*lane 4*) or 50 μmol/L LY 294002 for 24 hours. Fetuin-A (% w/v) was then added to the flasks: 0% (*lane1*), 0.25% (*lane 2*), and 0.5% (*lanes 3-5*). After 3 hours, the cells were harvested, lysed, and protein concentration of the lysates determined. The lysates were mixed with sample buffer and resolved in 10% SDS-PAGE. Following Western transfer, the blot was probed with antibodies against phospho-Akt473, phospho-Akt308, and total Akt, with acid stripping after each antibody.

background DBA/2, which is prone to calcification (10). This finding suggests an alternative explanation why fetuin-A null mutant mice are highly resistant to certain types of metastatic cancers. DBA/2/*Ahsg*$^{-/-}$ mice invariably develop extracellular calcification of most soft tissues. Tongue, kidney, myocardium, and lung tissue are most severely affected starting at 1 month of age. Interestingly, several malignancies are associated with reduced fetuin-A serum levels (37). This may be explained by the fact that fetuin-A in humans is one of the few negative acute phase proteins (i.e., depressed following trauma, infection or inflammation; refs. 1, 3, 4). Furthermore, tissue calcification is in fact diagnostic of various cancers like breast cancer and several life-threatening infective diseases like lung tuberculosis. Sequestration and immurement by calcification is a natural defense reaction of the body when confronted with invasive agents like cancer cells, mycobacteria, and other parasites. It is therefore tempting to speculate that soft tissue calcification is an ancient innate immune response to pathogen invasion. Fetuin-A–deficient mice are predisposed to soft tissue calcification and possibly have a calcification-prone or less permeable extracellular matrix, which is not permissive for metastatic cancer invasion. Although we cannot entirely rule out an indirect effect of fetuin-A on lung colonization as a result of calcification, the ability of acute injection of fetuin-A to reconstitute the effect suggests this is unlikely mechanism. However, the cancer-calcification link clearly merits further investigation.

In summary, we have shown here that fetuin-A, a serum protein is a potent stimulator of growth of LLC and possibly other metastatic cells in the liver, lungs, and s.c. regions of the body. The mechanism(s) suggested by our data is a novel growth signaling pathway mediated by fetuin-A and Ca^{2+} ions. This mechanism involves the PI3 kinase/Akt signaling and is likely to be one of the major growth pathways in tumor cells both *in vitro* and *in vivo*.

Acknowledgments

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The Serum Glycoprotein Fetuin-A Promotes Lewis Lung Carcinoma Tumorigenesis via Adhesive-Dependent and Adhesive-Independent Mechanisms

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