Journal of Cellular Biochemistry 101:1492–1504 (2007)

FGFR1/PI3K/AKT Signaling Pathway is a Novel Target for Antiangiogenic Effects of the Cancer Drug Fumagillin (TNP-470)

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Abstract Fibroblast growth factor-1 (FGF1), a prototypic member of the FGF family, is a potent angiogenic factor. Although FGF-stimulated angiogenesis has been extensively studied, the molecular mechanisms regulating FGF1-induced angiogenesis are poorly understood in vivo. Fumagillin, an antiangiogenic fungal metabolite, has the ability to inhibit FGF-stimulated angiogenesis in the chicken chorioallantoic membrane (CAM). In the current study, chicken CAMs were transfected with a signal peptide-containing version of the FGF1 gene construct (sp-FGF1). Transfected CAMs were then analyzed in the presence and absence of fumagillin treatment with respect to the mRNA expression levels and protein activity of the FGF1 receptor protein (FGFR1), phosphatidylinositol 3-kinase (PI3K), and its immediate downstream target, AKT-1 (protein kinase B). Treatment of sp-FGF1-transfected CAMs with fumagillin showed downregulation for both PI3K and AKT-1 proteins in mRNA expression and protein activity. In contrast, no major alterations in FGFR1 mRNA expression level were observed. Similar patterns of mRNA expression for the above three proteins were observed when the CAMs were treated with recombinant FGF1 protein in place of sp-FGF1 gene transfection. Investigation using biotin-labeled fumagillin showed that only the FGF1 receptor protein containing the cytoplasmic domain demonstrated binding to fumagillin. Furthermore, we demonstrated endothelial-specificity of the proposed antiangiogenic signaling cascade using an in vitro system. Based on these findings, we conclude that the binding of fumagillin to the cytoplasmic domain of the FGF1 receptor inhibited FGF1-stimulated angiogenesis both in vitro and in vivo. J. Cell. Biochem. 101: 1492–1504, 2007.

Key words: fumagillin; TNP-470; FGF; FGF1; FGFR1; PI3K; AKT; angiogenesis; antiangiogenesis; CAM

Angiogenesis is defined as the process of new blood capillary formation from existing ones. This process has been shown to be necessary for tumor growth and metastasis [Folkman, 1995]. The fibroblast growth factor (FGF) family is unique in its ability to stimulate various components of angiogenesis such as endothelial cell (EC) growth, migration, and blood vessel tube formation [Carmeliet, 2000; Partridge et al., 2000]. For this reason, FGFs are also thought to be key targets particularly in angiogenesis-dependent metastatic cancers [Friesel and Maciag, 1995]. In addition to being angiogenic factors, the FGF family members, in particular the two prototypes FGF1 (acidic FGF) and FGF2 (basic FGF), possess the ability to stimulate proliferation of a majority of mesoderm- and ectoderm-derived cells [Slavin, 1995; Cross and Claesson-Welsh, 2001]. In other words, the uninterrupted activity of FGF1 regulatory pathway in these cells is likely to lead to cancer progression characterized by abnormal growth and enhanced invasion of the tumor cells as well as an increase in tumor angiogenesis. In fact, others have demonstrated that both FGF1 and FGF2 as well as their shared high affinity receptor FGFR1 (*flg*) are upregulated and amplified in various solid tumors [Feng et al., 1997; Wang et al., 2002].

Grant sponsor: The Texas A&M University System HSC Funds; Grant sponsor: American Heart Association, Texas; Grant number: 0455045Y.

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Received 18 May 2006; Accepted 14 December 2006
DOI 10.1002/jcb.21265

Because tumor metastasis is dependent upon angiogenesis [Folkman, 1995], large efforts have been made to develop specific antiangiogenic drugs where the objective is to starve and thereby shrink tumor mass cells by eliminating their blood supplies. These efforts have resulted in the discovery of a number of highly profiled antiangiogenic compounds such as fumagillin (TNP-470) [Rosen, 2000], endostatin [O'Reilly et al., 1997], angiostatin [O'Reilly et al., 1994], VEGFR2 inhibitor PTK787/ZK 222584 [Drevs et al., 2000], EGFR inhibitors ZD1839 (Gefitinib, Iressa) [West et al., 2006] and OSI-774, (Erlotinib, Tarceva) [Hidalgo et al., 2002] and the inhibitor of c-Kit receptor tyrosine kinase imatinib mesylate (STI-571, Gleevec) [Millot et al., 2006] to name a few. We have been studying the molecular mechanisms regulating FGF1-induced angiogenesis and have been interested in understanding how fumagillin inhibits FGF1-induced angiogenic activity. Fumagillin is a fungal metabolite with potent antiangiogenic properties [Ingber et al., 1990; Antoine et al., 1994; Sin et al., 1997; Bhargava et al., 2000]. TNP-470, an analogue of fumagillin, is in a number of phase II clinical trials for the treatment of solid cancers [Rosen, 2000]. The current available data indicate that fumagillin exerts its antiangiogenic activity mainly by inhibiting the biological activity of methionine aminopeptidase (MetAP-2), a cytosolic enzyme that indiscriminately modifies newly synthesized cellular proteins by removal of the amino acid methionine positioned at their amino-termini [Klein and Folkers, 2003]. While there is strong published evidence supporting a role for the fumagillin mode of action through the annihilation of MetAP-2 activity [Klein and Folkers, 2003], we believe that the broad nature of substrates for MetAP-2—virtually any nascent protein in a cell—argues for the existence of a more specific target molecule/pathway for the antiangiogenic effects of fumagillin. For the purpose of our study, we have utilized the chicken chorioallantoic membrane (CAM) with high FGFR1 composition [Ribatti et al., 2001; Forough et al., 2003] as a model to observe angiogenesis in vivo. The major advantage of this model lies in its responsiveness to FGF stimulation in undergoing angiogenesis [Auerbach et al., 1991; Ribatti et al., 2001]; and that the angiogenic response can be blunted by fumagillin treatment [Ingber et al., 1990]. Among other advantages of this in vivo angiogenesis system are the immuno-compromised nature of embryos characterized by "take" of xenografts as well as its technical simplicity and cost-effectiveness [Auerbach et al., 1991; Ribatti et al., 2001]. Our recent studies with FGF1 treatment of the chicken CAM have shown that it is a useful system to elucidate signaling and other molecular pathways [Forough et al., 2005; Weylie et al., 2005].

In this study, we investigated the antiangiogenic activity of fumagillin on FGF1-stimulated angiogenesis in the chicken CAM. A cDNA encoding a secreted form of FGF1 (sp-FGF1) that had been previously shown to stimulate an exaggerated angiogenic response through activating the PI3K/AKT signaling cascade [Forough et al., 2005; Weylie et al., 2005] was transfected into the CAM model. Using this sp-FGF1-expressing angiogenic system, we sought to investigate whether the disruption of the FGF1/FGFR1/PI3K/AKT signaling pathway was responsible for the antiangiogenic mechanism of fumagillin.

MATERIALS AND METHODS

Chicken Embryos for Angiogenesis Assay

We previously described our chick CAM assay [Forough et al., 2003]. Briefly, single-comb-white-Leghorn fertilized chicken eggs (Poultry Sciences, Texas A&M University, College Station, Texas) were incubated at 37°C under an ambient atmosphere with constant humidity for 8 days. A rotary tool was used to create a circular opening of 6–8 mm in diameter in the shell over the air sac to expose the CAM.

Direct Gene Transfer of sp-FGF1 With and Without Fumagillin

A 50 μl mixture containing 10 μl (10 μg) of the sp-FGF1 expression plasmid or empty vector pMEXneo (for control) in methanol solvent; 10 μl (10 μg) of fumagillin (A.G. Scientific, Inc., San Diego, CA) in methanol solvent, 20 μl of Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA); and 10 μl of sterile PBS solution was pipetted onto the exposed CAM. The opening was sealed with parafilm and incubated at 37°C under constant humidity. CAMs were harvested 5 days post-transfection and digital images were prepared. For blood vessel counts, a "point counting" method was used [Forough et al., 2003]. After

digital images of the CAMs were produced, each group was transferred to a 1.5 ml microcentrifuge tube and stored at −20°C for further analysis.

For those experiments where rFGF1 was substituted for sp-FGF1 gene transfection, three doses of rFGF1 (50 ng at each time point/CAM; Biosource International, Inc.) were added directly to each CAM. Time points were days 8, 10, and 12.

CAM RNA Extraction

For RNA extraction from CAM tissue, we followed the protocol for Animal Tissue Samples included with the Totally RNA kit (Ambion, Inc., Austin, TX), a modified guanidinium thiocyanate-based method.

Real-Time PCR

For reverse transcription, 1 μg of total CAM RNA was incubated with 0.5 μg of random primers and 10 mM each dATP, dGTP, dCTP, dTTP in the presence of M-MLV RT, following the manufacturer's protocol (Invitrogen Life Technologies). For real-time PCR, each well contained a 50 μl mixture consisting of 3 μl of the CAM cDNA, 5 μl each of 300 nM primers specific to PI3K (forward: 5′-GGAATGAATGGCTGT-CGTATGAC-3′ and reverse: 5′-CCAATGGA-CAGTGCTCCTCT-TTA-3′), 25 μl Sybr Green 1 Mix (Eurogentec North America, Inc., Philadelphia, PA), and 12 μl dH_2O. Background controls substituted dH_2O for CAM cDNA. Baseline controls used chicken β-actin specific primers (forward: 5′-CTGATGGTCAGGTCATCACCATT-3′ and reverse: 5′-TACCCAAGAAAGATGGCT-GG-AA-3′) in substitution for PI3K, AKT-1 (forward: 5′-AAGGAAGGATGGCTCCACAAA-3′ and reverse: 5′-CGTTCCTTGTAGCCAAT-GAATGT-3′), and FGFR1 primers (forward: 5′-GAGACCACCTACTTCTCCGTCAAC-3′ and reverse: 5′-GGGATAGGT-CCAGTAAGGAGC-TACA-3′). Differences in mRNA levels were measured between sp-FGF1 with and without fumagillin treatment by quantitative amplification using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of gene expression was done using the Comparative Ct Method [Weylie et al., 2005].

CAM Tissue Protein Extraction

CAMs were thawed at room temperature (RT) and treated with 60 μl of Lysis buffer consisting 50 mM Tris HCl pH 7.5, 0.2% Tx-100, 10 mM $CaCl_2$ and kept on ice for 20 min. The samples were then centrifuged at 13,000*g* at 4°C for 10 min. The supernatant was then transferred to a fresh tube and the protein concentration determined using the BCA Protein Assay Kit (Pierce, Inc., Rockford, IL).

Western Blot Analysis of Phospho-AKT-1 and Total AKT-1

CAM protein was extracted from sp-FGF1, sp-FGF1 with methanol, pMEXneo vector, pMEXneo vector with methanol, sp-FGF1 with fumagillin, pMEXneo vector with fumagillin and fumagillin only samples. A volume containing 40 μg of protein from each sample was subjected to 10% SDS–PAGE and run at 200 V for approximately 45 min. The gel was then transferred to a nitrocellulose membrane (BioRad, Hercules, CA) and blocked in 5% BSA in TBST solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 40 min with gentle shaking at RT. After blocking, the membrane was probed with a 1:1,000 dilution of the primary antibody (phospho-AKT Ser 473, catalog #9271S; Cell Signaling Technology, Inc., Beverly, MA) overnight with shaking at 4°C. The membrane was washed three times for 5 min each in TBST to remove non-specific primary antibody binding and then probed with a 1:25,000 dilution of HRP-conjugated donkey anti-rabbit IgG secondary antibody (Pierce, Inc.) made in TBST containing 5% BSA for 1 h at RT. The membrane was washed three times with TBST for 10 min and processed for development using SuperSignal West Pico Chemiluminescent kit (Pierce, Inc.) according to the manufacturer's recommendations. The membrane was then exposed to film, the luminescent image scanned into a computer, and analyzed for optical density using the Multianalyst software program (BioRad, Philadelphia, PA). After the signals on the membranes were quantified, they were stripped of the phospho-AKT antibody by incubating for 30 min at 70°C with shaking in a stripping buffer consisting of 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol. The membrane was washed in TBST wash buffer three times for 5 min each and re-probed with total AKT-1 antibody (Catalog #9272; Cell Signaling Technology, Inc.) for loading control.

Fumagillin Biotinylation

Fumagillin (1.0 mg) (Catalog # F-1028, A.G. Scientific, Inc.) was first dissolved in 500 μl of methanol as recommended by the manufacturer. Separately, biotin (EZ-Link Biotin-PEO-Amine, Pierce, Inc.) was dissolved in MES buffer (2-N-morpholino ethanesulfonic acid, pH 5.0) at a concentration of 50 mM. Equal volumes of the biotin solution was then added to the diluted fumagillin solution and mixed. Fifty microliters of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, Pierce, Inc.) in MES buffer at 20 mg/ml was then added to the solution and mixed. The resulting mixture was incubated for 2 h at RT with stirring. Non-reacted biotinylation reagent and EDC by-product from the biotinylated fumagillin was removed by dialysis overnight at 4°C in 10 mM Tris buffer pH 7.

Immunoprecipitation of Biotin-Fumagillin With FGF1 Receptor Protein

In a microcentrifuge tube, 2.5 μg recombinant human FGFR1 protein containing amino acids 456–765 (Catalog #14-582, Upstate Cell Signaling Solutions, Inc., Lake Placid, NY) was solubilized in PBS buffer (0.1 M phosphate, 0.15 M NaCl; pH 7.0). Dialyzed fumagillin-biotin was added and the mixture incubated overnight at 4°C with shaking. Re-suspended beaded agarose cross-linked with avidin (Immobilized NeutrAvidin, Pierce, Inc.) was combined with the protein/biotinylated fumagillin mixture. Sample was incubated with mixing for 1 h at RT. After incubation, sample was washed four times with 0.5 ml of PBS buffer and centrifuged for 1–2 min at ~2,500*g* at RT. Supernatant was removed from the final wash and the sample boiled in SDS–PAGE sample buffer prior to electrophoresis.

Cell Culture Study

Primary ECs isolated from 15 μm diameter post-capillary venules of the bovine heart [Schelling et al., 1988] were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO/BRL; Grand Island, NY) containing high glucose and L-glutamine supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone; Logan, UT), 100 U/ml penicillin G and 100 μg/ml streptomycin (GIBCO/BRL; Grand Island, NY). The authenticity of ECs was demonstrated by their unique ability to uptake acetylated low-density lipoprotein (LDL) (data not shown). Approximately 1×10^4 ECs were seeded per well of a 6-well tissue culture plate in triplicate in 10% FBS/DMEM and incubated at 37°C with 5% CO_2/95% air overnight. Next day, the medium was removed from wells and cells washed with PBS. Medium was replaced with fresh 0.5% FBS/DMEM and incubated for 48 h. Following this incubation, the cells were treated with rFGF1 protein alone (10 ng/ml), fumagillin alone (10 μg/ml), or rFGF1 plus fumagillin. For cell number determinations, cells were harvested by trypsinization at different time points and counted using a hemacytometer.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the Student's *t*-test. All results are expressed as mean ± SD. $P < 0.05$ was accepted as significant.

RESULTS

Fumagillin Inhibits Vascularization in sp-FGF1 CAMS

We previously reported the construction and over-expression of a secreted version of the angiogenic growth factor FGF1 (sp-FGF1) in the chicken CAM [Forough et al., 2003]. Subsequently, we identified that the angiogenic response triggered by sp-FGF1 over-expression is in part mediated via the PI3K/AKT signaling pathway [Forough et al., 2005; Weylie et al., 2005]. We sought to investigate whether fumagillin inhibits FGF-induced angiogenesis by influencing the PI3K/AKT signaling pathway. Based on our initial dose response curve study (n = 3 eggs per drug concentration), an ideal dose of 10 μg fumagillin was determined to inhibit sp-FGF1-mediated angiogenesis without toxicity in CAMs (data not shown). The degree of angiogenesis was measured between sp-FGF1 (10 μg) and sp-FGF1 plus fumagillin (10 μg) treated CAMs (Fig. 1). Based on these studies, we observed a 30% reduction in angiogenesis in sp-FGF1 cDNA transfected CAMs 5 days after treatment with fumagillin ($P < 0.05$).

Fumagillin Alters mRNA Levels of PI3K in sp-FGF1 CAMs

We next sought to evaluate fumagillin's effect on the PI3K pathway due to our recent data supporting the importance of this pathway in

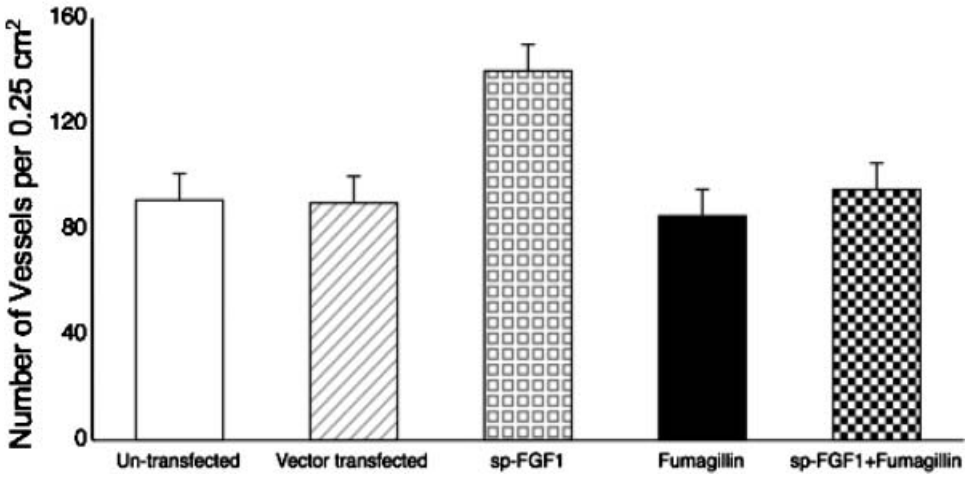

Fig. 1. Number of CAM vessels counted per 0.25 cm^2 in Days 5 post-gene transfection. CAMs were transfected with 10 μg of sp-FGF1 cDNA construct or 10 μg of sp-FGF1 construct in the presence of 10 μg fumagillin. All samples were incubated for 5 days at 37°C under ambient atmosphere. Control samples were either un-transfected or transfected with the empty vector pMEX neo. Eggs were sacrificed 5 days post-treatment and the CAM tissues removed to collect digital images and to count vessels. (n = 5 eggs per group, $*P < 0.05$, Student's *t*-test for unpaired samples.)

FGF1-stimulated angiogenesis in chicken CAM [Weylie et al., 2005]. To determine whether fumagillin influenced its antiangiogenic activity via the PI3K signaling pathway, we assessed alterations in mRNA levels of p85α, the regulatory subunit of PI3K Class IA. Class IA PI3K is known to mediate downstream signaling of the activated receptor tyrosine kinases [Krymskaya et al., 2001]. Using RT-real-time PCR, we showed over twofold increase in PI3K p85α mRNA level in sp-FGF1 cDNA transfected CAMs at day 5 post-transfection (Fig. 2). Treatment with fumagillin inhibits sp-FGF1-stimulated p85α mRNA synthesis ($P < 0.05$).

Fumagillin Alters mRNA Levels of AKT-1 in sp-FGF1 CAMs

Since it has been documented that PI3K often partners with AKT-1 in signaling cascades [Cantley, 2002], we assessed whether fumagil-

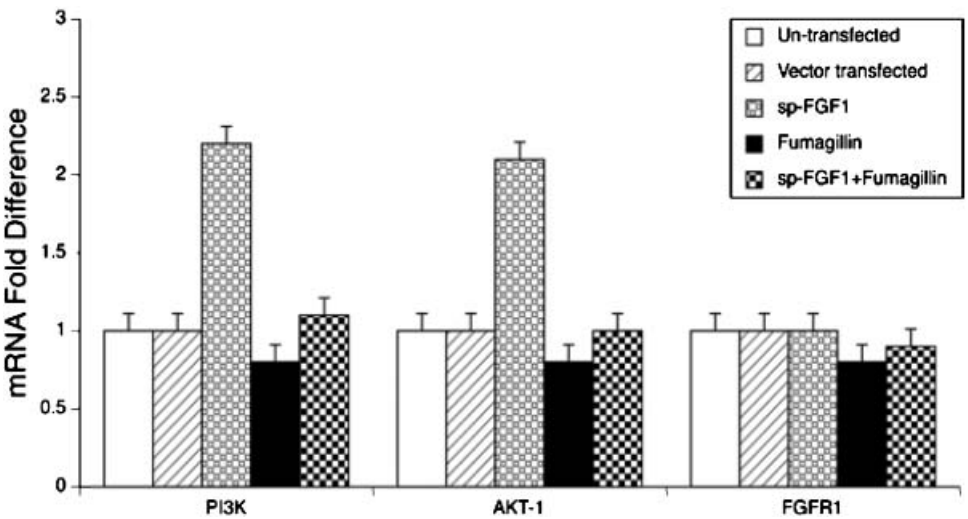

Fig. 2. Quantitative PCR of mRNA levels in day 5 CAMs. According to this data, sp-FGF1 alone-treated samples exhibit a twofold increase in mRNA expression levels of PI3K and AKT-1. However, FGFR1 mRNA expression level remains the same in tested conditions. Chicken β-actin served as internal control. Experiments were performed in triplicate and at least two independent experiments were performed. ($*P < 0.05$, Student's *t*-test for unpaired samples.)

lin treatment alters mRNA synthesis of AKT-1 in our chicken CAM model of angiogenesis. We chose to focus on day 5 CAMs only since we had established significant reductions in both vessel numbers (Fig. 1) and PI3K mRNA synthesis in response to fumagillin treatment for this time point. Our RT-real-time PCR evaluation of AKT-1 revealed a statistically significant decrease (>2-fold) in AKT-1 mRNA level in sp-FGF1 plus fumagillin compared to sp-FGF1 only treated CAMs (Fig. 2).

Fumagillin Does Not Alter mRNA Level of FGF1 Receptor in sp-FGF1 CAMs

Since FGF1 exerts its biological effects through its high affinity receptor FGFR1, and because FGFR1 is a likely target for fumagillin binding and antagonism, we evaluated the effect of fumagillin on FGFR1 mRNA levels. RT-real-time PCR was employed to assess the FGFR1 mRNA levels. We observed no significant differences when comparing the level of mRNA expression between sp-FGF1-treated and sp-FGF1 + fumagillin-treated CAMs (Fig. 2). These data show that fumagillin does not downregulate the FGFR1 gene expression when suppressing angiogenesis.

Fumagillin Alters AKT-1 Protein Activity in sp-FGF1-Treated CAMs

In order to confirm the activity of FGF1 acting through the PI3K/AKT signaling pathway, we measured changes in phospho-Akt formation since phosphorylation of AKT-1 has been used as an indicator for the activation of the PI3K/AKT pathway by our lab [Forough et al., 2005; Weylie et al., 2005] and others [Nakashio et al., 2002]. Our data showed that sp-FGF1-stimulated AKT-1 phosphorylation decreased threefold compared to sp-FGF1 only treated CAMs (Fig. 3) ($P < 0.05$). These results suggest that fumagillin exerts its antioangiogenic activity by downregulating FGF-triggered PI3K/AKT signal transduction.

Fumagillin Inhibits Vascularization in rFGF1 CAMs

We substituted sp-FGF1 gene transfection in our CAM assay with pure recombinant FGF1 protein (rFGF1) in order to obtain a better estimation of: (1) the concentration of FGF1 protein required to stimulate angiogenesis, (2) fumagillin's antiangiogenic effect via pure FGF1-stimulated angiogenesis. Similar to the previously described experiment with sp-FGF gene transfection, we observed a reduction in CAM angiogenesis albeit to a lesser extent (Fig. 4). These data demonstrate that fumagillin exerts its antiangiogenic activity on FGF1-stimulated angiogenesis regardless of whether FGF1 is provided as a transgene or pure protein.

Fumagillin Alters mRNA Levels of PI3K and AKT-1 But Not FGFR1 in rFGF1 CAMs

To confirm that irrespective of the delivery method of FGF1 into the chick CAM and that fumagillin inhibits angiogenesis via

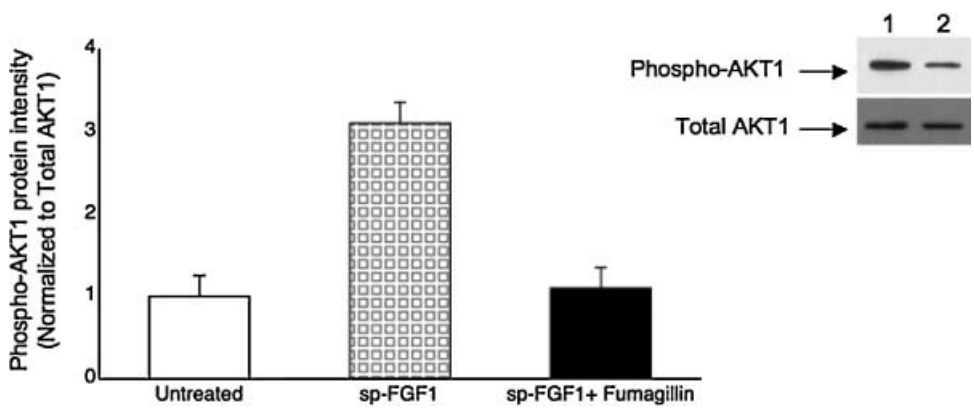

Fig. 3. Western blot protein analysis using a phospho-specific AKT-1 antibody to detect PI3K phosphorylated AKT-1 in sp-FGF1 and sp-FGF1 plus fumagillin treated CAMs. There is a significant decrease in the amount of phospho-AKT-1 in sp-FGF1 plus fumagillin (**Lane 2** top) versus sp-FGF1 only (**Lane 1** top) samples. Total AKT-1 antibody was used to quantify total AKT-1 protein in each sample (n = 3 egg for each sample). Three independent experiments were performed, $^*P < 0.05$, Student's *t*-test for unpaired samples.

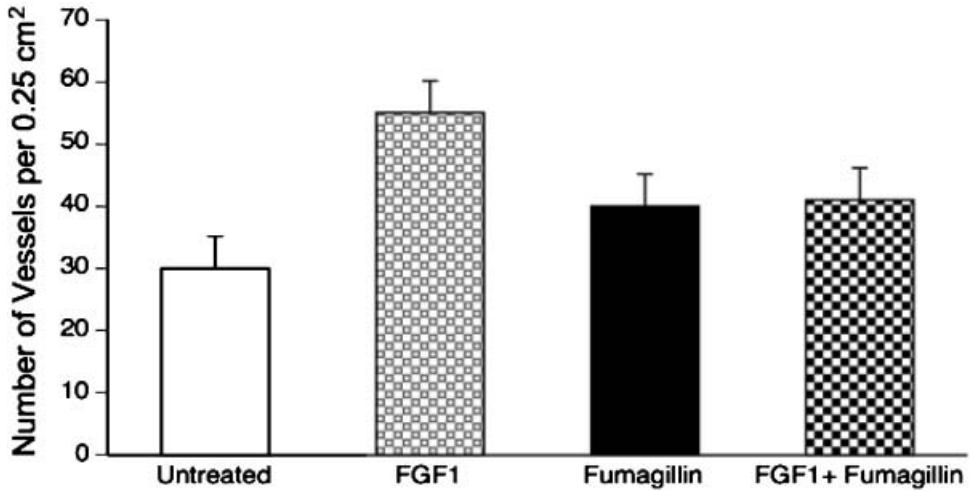

Fig. 4. Number of CAM vessels counted per 0.25 cm^2 in day 5 post-recombinant FGF1 application. CAMs were applied with 50 ng/CAM rFGF1 plus 10 μg/CAM fumagillin and incubated for 5 days at 37°C under ambient atmosphere. Starting on day 5, eggs were sacrificed and the CAM tissues removed to collect digital images and to count the vessels. (n = 5 per group, $*P < 0.05$, Student's *t*-test for unpaired samples.)

the FGFR1/PI3K/AKT signaling pathway, we assessed alterations in mRNA levels of PI3K p85α, AKT-1, and FGFR1 in CAM tissues in response to rFGF1 treatment. We detected similar patterns of gene expression for these molecules when comparing to sp-FGF1-treated CAMs. In other words, there is a statistically significant decrease in PI3K (twofold) and AKT-1 (threefold) but not FGFR1 mRNA levels in rFGF1 protein plus fumagillin compared to rFGF1 alone treatments using RT-real-time PCR (Fig. 5). Taken together, these data suggest: (i) a sustained supply of FGF1 is required to stimulate angiogenesis as shown by the sp-FGF1 gene transfection approach which resulted in constitutive generation of the FGF1 protein; and also the application of pure rFGF1 protein in three deliveries that span the course of the study, (ii) the effect of fumagillin on the FGFR1/PI3K/AKT pathway is long-lasting and can be detected at least 5 days following exposure.

Fumagillin Exhibits Binding to FGF1 Receptor Using a Cell-Free System

Given the apparent role of the FGF receptor in fumagillin action, it is important to establish whether fumagillin was acting directly or

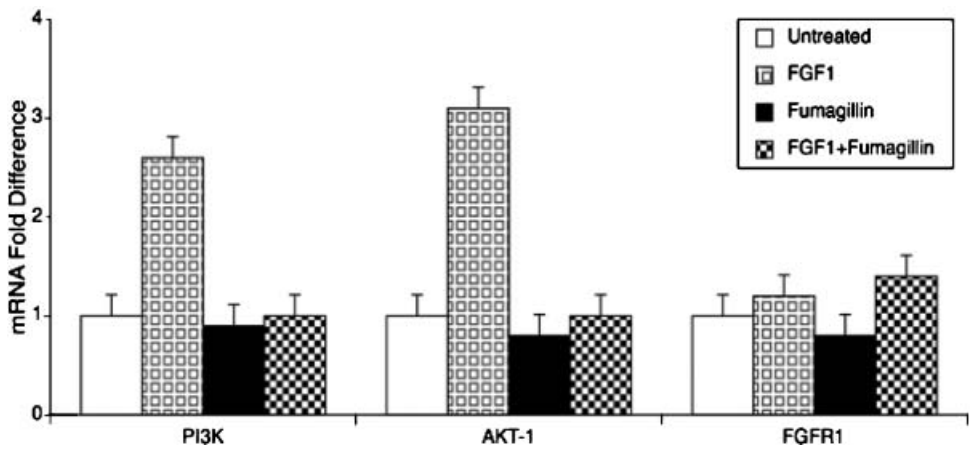

Fig. 5. RT-real-time PCR of rFGF1-treated CAMs shows elevated mRNA levels of PI3K, AKT-1, but not FGFR1. The evaluated mRNA levels were normalized to actin mRNA levels in each condition. Experiments were performed in triplicates and at least two independent experiments were performed. ($*P < 0.05$, Student's *t*-test for unpaired samples.)

indirectly to alter FGF receptor-mediated responses. To determine if fumagillin binds to FGFR1, we designed a cell-free system where we assessed the ability of a pure recombinant fusion protein consisting of the FGFR1 intracellular domain encompassing amino acids 456–765 linked to a GST tag to bind to biotin-labeled fumagillin. Precipitation of the bound protein using avidin-conjugated agarose beads followed by Western blotting to detect the GST tag, revealed a protein of ~72 kDa (Fig. 6). The increase in size can be accounted for by the aggregate molecular weights of fumagillin, biotin, and the cross-linking agent EDC (Fig. 6). Furthermore, the specificity of fumagillin for FGFR1 was substantiated as the biotinylated fumagillin failed to bind and co-immunoprecipitate with pure recombinant proteins PI3K, AKT-1, and GST (data not shown). These data support the hypothesis that fumagillin can bind to the intracellular domain of the FGFR1 protein.

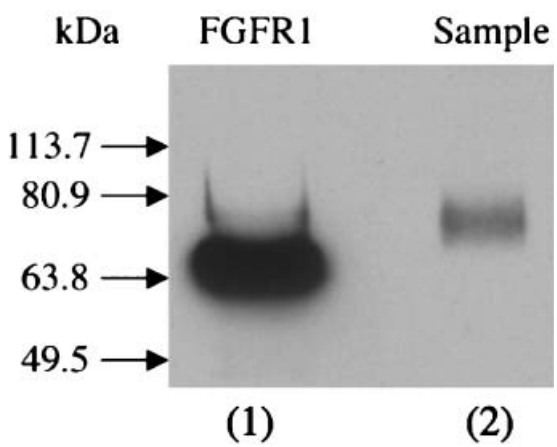

Fig. 6. In vitro binding of biotinylated fumagillin to a human recombinant FGFR1 protein. A higher molecular weight complex is observed when a biotinylated fumagillin substrate was incubated with pure recombinant FGFR1 protein (1/12 ratio of bound protein to total protein in sample, as determined by density analysis using the Multianalyst software program). Precipitation of the bound complex using avidin-conjugated agarose beads was followed by Western blotting using antibody to the GST tag linked to the FGFR1 protein (**Lane 2**). Pure recombinant FGFR1/GST fusion protein was Western blotted to show the shift in molecular weight of fumagillin complex and serves as a positive Western blot control (**Lane 1**). Specificity of fumagillin for FGFR1 was confirmed as biotin-labeled fumagillin did not exhibit binding to pure recombinant proteins PI3K, AKT-1, or GST when tested in a similar immuno-precipitation/Western assay (data not shown). The gel is representative of three independent experiments.

Fumagillin Inhibits FGF1-Stimulated Growth of Cultured Endothelial Cells (ECs)

To confirm that fumagillin acts directly on ECs, we investigated the growth-inhibitory effect of fumagillin on FGF1-stimulated bovine microvascular ECs grown in culture. A combination of fumagillin plus rFGF1 or fumagillin alone treatment exhibited approximately 5- to 10-fold decrease in cell number compared to rFGF1 alone-stimulated ECs at 3 and 7 days post-treatment, respectively (Fig. 7). These data demonstrate that fumagillin directly targets ECs and inhibit cell proliferation in both FGF1-dependent and FGF1-independent fashion.

Fumagillin Alters mRNA Levels of PI3K and AKT-1 But Not FGFR1 in rFGF1-Stimulated Cultured ECs

To demonstrate that fumagillin also acts on the FGFR1/PI3K/AKT signaling pathway in vitro, we assessed mRNA levels of FGFR1, PI3K, and AKT-1 as before. Similar to our observations in vivo, we detected a statistically significant decrease in PI3K (13-fold) and AKT-1 (4-fold) but not FGFR1 mRNA levels in rFGF1 plus fumagillin samples compared to rFGF1 only treated ECs (Fig. 8).

DISCUSSION

Results of the present study reveal several important findings with broad implications in the field of therapeutic angiogenesis: (1) a novel molecular mechanism for the action of fumagillin in inhibition of angiogenesis is elucidated, (2) fumagillin and its less-toxic analogues may serve as excellent specific anticancer drugs where FGF-dependent tumor angiogenesis is the main cause of tumor progression, (3) fumagillin may be an effective anticancer drug for other receptor tyrosine kinase family members with close structural resemblance to FGFR1.

This study is a continuation of our previous efforts in demonstrating the importance of the PI3K/AKT signaling pathway in regulating FGF1-induced angiogenesis in vivo [Weylie et al., 2005]. In the current study, we have demonstrated the novel action of fumagillin on the PI3K/AKT pathway leading to inhibition of FGF1-induced angiogenesis in vivo and inhibition of endothelial cell proliferation in vitro.

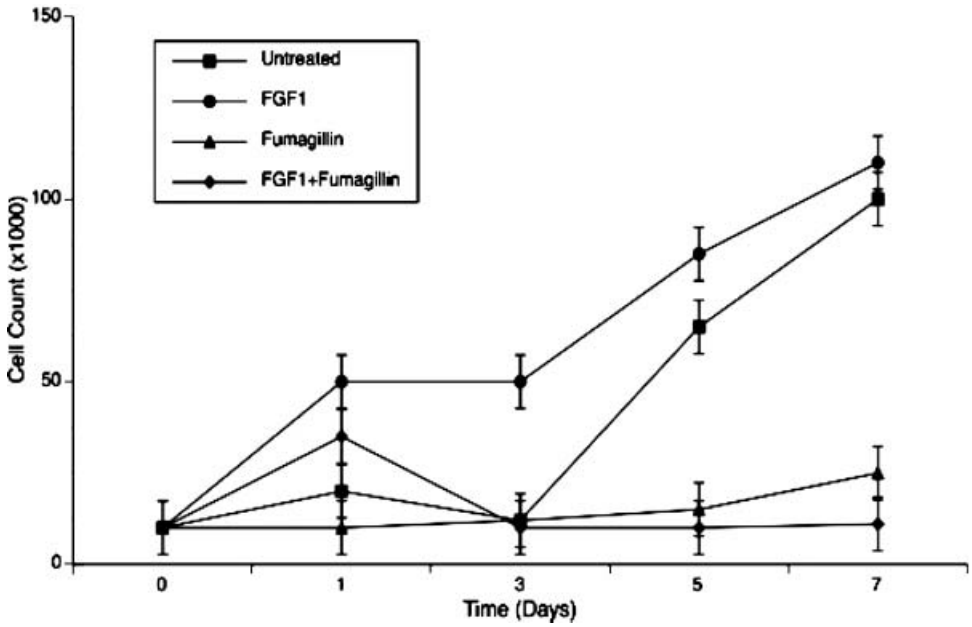

Fig. 7. Inhibition of cultured endothelial cell proliferation in response to fumagillin treatment. At the designated time points, cells were trypsinized and counted. Results are shown as mean ± SD for triplicate determinations. At least four independent experiments were performed.

Angiogenesis plays a pivotal role in tumor growth and metastases. The inhibition of angiogenesis is emerging as a promising new strategy for the treatment of cancer. Fumagillin is a commercially available compound with potent anti-angiogenic activities. It has been shown in various in vivo and in vitro studies to have an inhibitory effect on the proliferation of endothelial cells [Pyun et al., 2004; Mazzanti et al., 2004]. TNP-470, a synthetic analogue of fumagillin is currently undergoing clinical trials for a variety of cancers [Bhargava et al., 2000]. The molecular mechanism governing fumagillin's antiangiogenic effects is still not completely understood and is actively being investigated by various research laboratories.

Fig. 8. RT-real-time PCR of rFGF1-treated endothelial cells shows significant elevation of mRNA levels of PI3K, AKT-1, but not FGFR1. The evaluated mRNA levels were normalized to actin mRNA levels in each condition. Experiments were performed in triplicates and at least three independent experiments were performed.

Several studies have shown that fumagillin interferes with endothelial cell proliferation by inhibiting cell-cycle progression [Antoine et al., 1994] and others have demonstrated its inhibitory effects occur via inhibition of methionine aminopeptidase 2, a cytosolic enzyme involved in maturation of nascent proteins inside the cell, by acting on their amino-terminal methionine [Griffith et al., 1997; Sin et al., 1997].

In the current study, we present data that fumagillin inhibits FGF1-induced angiogenesis through influencing the FGF1/FGFR1/PI3K/AKT pathway. We have reached this conclusion based on the following observations: (1) FGF1-stimulated chicken CAM that was simultaneously co-treated with fumagillin significantly reduced mRNA expression levels of PI3K and AKT-1 genes but not that of FGFR1, (2) fumagillin-inhibited proliferation of cultured ECs and demonstrated reduction in EC mRNA expression levels of PI3K and AKT-1 but not FGFR1 genes, (3) fumagillin demonstrated strong binding to recombinant FGFR1 (cytosolic domain) in a cell-free system but not to either recombinant PI3K p85α subunit or AKT-1 proteins (data not shown), (4) in addition to downregulating mRNA expression levels of PI3K and AKT-1 genes, fumagillin treatment of FGF1-stimulated CAM significantly inhibited PI3K activity as demonstrated by reduction in AKT-1 phosphorylation.

Despite the known role of FGF1 as an inducer of angiogenesis, its signaling through the PI3K pathway has not been well examined as a potential target for therapeutic angiogenesis. Based on our recent findings regarding the importance of the PI3K signaling pathway in mediating FGF1-triggered angiogenesis, we decided to evaluate whether the inhibitory mechanism of fumagillin involves PI3K/AKT signaling cascade.

Utilizing the chicken CAM as our experimental model, we examined how fumagillin may have exerted its antiangiogenic properties on the morphology of the vessel vasculature as well as key players within the PI3K signaling pathway including the FGF1 receptor protein, PI3K, and the AKT-1 proteins. Our results showed that fumagillin co-administered with FGF1 (transgene or pure protein) significantly decreased the mRNA expression levels of both PI3K and AKT-1 (Fig. 2). Furthermore, the activity of AKT-1 correlated with its expression pattern as demonstrated by a significant decrease in the level of phospho-AKT-1 (active form of AKT-1) in fumagillin plus sp-FGF1 compared to sp-FGF1 alone treated CAMs (Fig. 3). Interestingly, we did not detect any significant changes in the mRNA levels of FGFR1 when comparing fumagillin plus sp-FGF1 to sp-FGF1 alone CAMs (Fig. 2). One interpretation of this latter observation is that fumagillin might have the ability to bind the FGFR1 protein and in the process downregulate the downstream PI3K/AKT signaling required for angiogenesis in the CAM. Although one cannot predict the exact location on FGFR1 where fumagillin might bind to in order to exert its antiangiogenic action, we chose to assess fumagillin's binding ability to the cytoplasmic domain of the FGFR1 protein. This decision was based on two reported modes of action for fumagillin, including the targeting of the MetAP-2 enzyme and a cell-cycle control protein, both of which are cytosolic proteins [Antoine et al., 1994; Klein and Folkers, 2003]. In our approach to investigate the relationship between fumagillin and a recombinant protein consisting of the FGFR1 cytosolic domain fused to a GST tag, we biotinylated fumagillin and tested whether a complex of fumagillin-FGFR1 can be extracted from solution using avidin cross-linked to agarose beads. The bead-bound materials were subjected to Western analysis using antibody specific to the GST moiety. Our finding shows the presence of a higher molecular complex that has been shifted relative to the control sample containing purified FGFR1/GST fusion protein (Fig. 6). We believe that this higher molecular weight complex is the result of the binding of the biotin-fumagillin moiety to the FGFR1 protein. The prediction from our study is that fumagillin binds to the cytoplasmic domain of the FGF1 receptor protein thereby blocking the phosphorylation of the tyrosine residues, positioned on the FGFR1 within the kinase domain, that are necessary to initiate the downstream signaling events. The purified, recombinant human FGFR1/GST fusion protein employed in this study contained only amino acid residues 456–765 which is located within the C-terminal domain of the receptor. Within this domain are the five key tyrosine residues 583, 585, 653, 654, and 730 that are required to initiate a signaling event when phosphorylated. Activation of the PI3K protein requires a phosphorylated tyrosine residue on the receptor which serves as a docking site for

the p85α regulatory subunit of PI3K [Chang et al., 2003]. This then recruits the catalytic subunit of PI3K, p110, to this complex. However, if the site containing the tyrosine residues were blocked, such as by fumagillin, then PI3K binding cannot occur and no signaling event can be initiated. We believe that fumagillin selectively binds to the cytoplasmic domain of the FGFR1 protein and thus "shut off" upstream signaling to the PI3K/AKT signaling pathway. Fumagillin is a relatively small molecule (459 Da) that can readily diffuse across the cytoplasmic membrane and bind to intracellular proteins.

Our data correlate well with our conclusion that by inhibiting early signaling at the cell surface, activation of all downstream intermediates within the signaling cascade will be affected. This explains the downregulation of mRNA and protein expression levels of PI3K and AKT-1 without affecting the FGFR1 mRNA expression.

We also performed a computational search to determine whether there are other known proteins with strong sequence homology to the cytoplasmic domain of the FGFR1 protein used in our study. The rationale was that these cellular proteins may also serve as potential targets for interaction with fumagillin and perhaps play a role in fumagillin-mediated inhibition of angiogenesis. Remarkably, our search identified a number of proteins—all receptor tyrosine kinases—that demonstrated high sequence identity (>70%) with a stretch of 20 amino acids ASKKCIHRDLAARNVLVTED located within the intracellular domain of the FGFR1 protein. These RTKs include VEGFR-2, also known as Flk-1 (78% identity); human KIT protein (83% identity); VEGFR-1 or Flt-1 (73% identity); megakaryocyte-associated tyrosine-protein kinase, also known as MATK (78% identity); c-Src tyrosine kinase (86% identity); tyrosine kinase JAK3 (75% identity); and the LsK protein (73% identity). The results from this search raise two very exciting questions with major implications in the field of therapeutic angiogenesis. First, does fumagillin also have the ability to bind to the cytosolic domain of VEGFR1 and 2 and in the process inhibit VEGF-stimulated angiogenesis? Second, does fumagillin also have the ability to inhibit bone marrow-derived progenitor endothelial cells? The possibility of the latter is based on (i) that some of the above protein with high sequence identity to FGFR1 are present in the bone marrow stem and progenitor cells, and (ii) a report that fumagillin inhibits hematopoiesis [Hasuike et al., 1997].

There has been one published report documenting the binding of fumagillin to low affinity FGF receptors [Bond et al., 2000]. However, no reports so far have shown fumagillin binding to any of the four high affinity FGF receptors including FGFR1, FGFR2, FGFR3, FGFR4, or any of their splice variants. High affinity FGFR1 through four are the products of genes on different chromosomes [Werner et al., 1992] and serve as the main receptors for FGF-stimulated angiogenesis. We predict that the other three high affinity FGFRs also have the ability to bind to fumagillin since our BLAST search showed that the intracellular domain of FGFR1 used in this study has more than 85% homology to the three high affinity FGFRs.

Our findings show that fumagillin-alone treatment of ECs also demonstrated potential anti-proliferative effects. This observation does not only rule out the possibility that fumagillin acts through the FGFR1/PI3K/AKT signaling pathway but also implies that fumagillin's inhibitory effect can be mediated by unrelated signaling cascades as proposed by others [Liu et al., 1999; Zhang et al., 2006].

Besides the PI3K/AKT signaling pathway, several other pathways can also be regulated by phosphorylation of the FGFR's cytoplasmic domain. Fumagillin's binding to this region can potentially have an effect on the regulation of these downstream pathways. In fact, preliminary data suggest that the JNK pathway (involved in cell apoptosis) is negatively regulated as a result of fumagillin binding while the ERK pathway (involved in cell growth and differentiation) is not affected (data not shown).

In summary, we have introduced a new pathway for fumagillin's antiangiogenic function. Our future plan is to test whether fumagillin uses the same FGFR1/PI3K/AKT pathway to inhibit FGF1-induced angiogenesis in mammalian systems of angiogenesis and tumor angiogenesis.

ACKNOWLEDGMENTS

We thank Dr. Paul Brandt, Texas A&M University Health Science Center, for his scientific advice and helpful coments on the manuscript. This work was supported by The

Texas A&M University System HSC Funds and Research Grant 0455045Y from the American Heart Association, Texas Affiliate (RF).

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