

Inhibition of MAPK Kinase Signaling Pathways Suppressed Renal Cell Carcinoma Growth and Angiogenesis *In vivo*

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Abstract

The mitogen-activated protein kinase (MAPK) signaling pathways play essential roles in cell proliferation and differentiation. Recent studies also show the activation of MAPK signaling pathways in tumorigenesis, metastasis, and angiogenesis of multiple human malignancies, including renal cell carcinoma (RCC). To assess the role of this pathway in regulating the proliferation and survival of RCC cells, we first examined the expression of MAPK kinase (MKK) and MAPK in clear cell RCC and confirmed the overexpression of *MKK1* and extracellular signal-regulated kinase 2 (*ERK2*) in these tumors. We then tested the effects of pharmacologic inhibition of MKK on human RCC cell lines, both *in vitro* and *in vivo*, using anthrax lethal toxin (LeTx), which cleaves and inactivates several MKKs. Western blotting showed that the phosphorylation levels of ERK, c-Jun-NH₂ kinase, and p38 MAPK decreased after 72 h of LeTx treatment. Exposure to LeTx for 72 h reduced cell proliferation by 20% without significant effects on cell cycle distribution and apoptosis. Anchorage-independent growth of RCC cells was dramatically inhibited by LeTx. *In vivo* studies showed that tumor growth of RCC xenografts could be suppressed by LeTx. Extensive necrosis and decreased tumor neovascularization were observed after LeTx treatment. LeTx also showed direct inhibition of proliferation of endothelial cells *in vitro*. Our results suggest that suppression of one or more MAPK signaling pathways may inhibit RCC growth through the disruption of tumor vasculature. [Cancer Res 2008;68(1):81–8]

Introduction

Renal cell carcinoma (RCC) arises in the renal cortex and comprises 80% to 85% of all kidney cancers. Although it only accounts for ~2% of all cancers, the incidence of kidney malignancies has increased over the past three decades. Annually, there are over 36,000 newly diagnosed cases in the United States and nearly 210,000 new cases worldwide. Mortality is ~12,600 deaths each year in the United States and 102,000 deaths

worldwide. Five-year survival rates for localized, regional, and distant disease are 90%, 60%, and 9%, respectively (1, 2). RCC is refractory to chemotherapy and radiotherapy. Encouragingly, two targeted therapeutics, sunitinib (Sutent) and sorafenib (Nexavar), which target multiple receptor tyrosine kinases and Raf kinases, have shown better response than other agents in clinical trials and have recently been approved to treat advanced human RCC (3–5).

Mitogen-activated protein kinase (MAPK) kinases (MKK) are crucial enzymes at the intersection of several biological pathways that regulate cell differentiation, proliferation, and survival (6, 7). In response to a variety of extracellular stimuli, MKKs become activated and then phosphorylate MAPKs, including extracellular signal-regulated protein kinase (ERK), c-Jun-NH₂ kinase (JNK), and p38 MAPK (p38). Mutations of proteins in this pathway have been found to contribute to ~20% of all human cancers (8, 9). MKK/ERK signaling has been shown to play a crucial role in tumorigenesis and tumor metastasis (10–12). Activated ERK or elevated ERK expression has been detected in a variety of human tumors, including breast carcinoma, glioblastoma, and primary tumor cells derived from kidney, colon, and lung tissues (13–16). Sustained activation of ERK has been established as a requirement for angiogenesis (17–21). Overexpression of MKK was also found in human RCC cases (52%; ref. 22).

In this study, we first found significantly high expression of *MKK1* and *ERK2* in human clear cell RCC relative to normal controls. We therefore hypothesize that targeting this pathway may be of therapeutic value against ccRCC. To test this hypothesis, we used anthrax lethal toxin (LeTx) as an inhibitor to block multiple MAPK signaling pathways. LeTx is an exotoxin produced by the Gram-positive bacterium *Bacillus anthracis* and comprises two proteins: protective antigen and lethal factor. Protective antigen, by itself, is not toxic; it serves to translocate lethal factor to the cytosol. Lethal factor is a Zn²⁺-metalloprotease, which specifically cleaves the NH₂-termini of MKK1, MKK2, MKK3, MKK4, MKK6, and MKK7, but not MKK5 (23–25). Cleavage results in loss of kinase function of MKKs (26). It has been shown that LeTx reverts the transformation of V12 H-ras-transformed NIH 3T3 cells *in vitro* and inhibits their growth and vascularization *in vivo* (27). Inhibition of MAPK signaling with either LeTx or a small-molecule MKK inhibitor triggers apoptosis in human melanoma cells (28, 29). LeTx also causes tumor regression in human melanoma xenograft models (28, 30).

In summary, in this paper, we show that there is an overexpression of MKK and MAPK in clear cell RCC, and inhibition of MKK signaling by LeTx suppresses RCC growth, as well as disrupts tumor vascularization *in vivo*.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-5311

Materials and Methods

Reagents. Protective antigen, lethal factor, and E687C (an inactive form of lethal factor) were purified from cultures of *B. anthracis*, as described (31). U0126 was purchased from Calbiochem (EMD Biosciences) and prepared as 10 mmol/L stock solution in DMSO.

Expression of MKK and MAPK in clear cell RCC. The mRNA expression of MKK-related and MAPK-related genes was measured in 174 clear cell renal tumors and 15 normal kidney samples using Affymetrix HGU133 Plus 2.0 microarrays, as described elsewhere (32).

Cells and cell culture. ACHN, A-498, 786-O, 769-P, SW156, Caki-1, and Caki-2 RCC cell lines were obtained from the American Type Culture Collection. UO-31, TK-10, SN12C, and RFX393 cells were kindly provided by Dr. George Vande Woude (Van Andel Research Institute); SKRC39 cells were obtained from Memorial Sloan-Kettering Cancer Center. The cells were maintained in DMEM or RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) in a humidified incubator containing 5% CO₂ at 37°C. Human umbilical vascular endothelial cell (HUVEC) were obtained from Clonetics and maintained in Clonetics EBM-2 medium supplemented with EGM-2 singlequots (Cambrex).

Cell proliferation assay

(a) Cell growth. Cells were seeded in 24-well plates (8,000 cells per mL) 24 h before initiation of treatment with LeTx (1 µg/mL protective antigen plus 0.5 µg/mL lethal factor) or U0126 (10 µmol/L from 10 mmol/L stock solution in DMSO) for 8 days. E687C (0.5 µg/mL) plus protective antigen (1 µg/mL), 0.1% DMSO, and untreated cells served as controls. Medium containing inhibitors was replaced every 3 days during each experiment. Each day, one plate of cells was trypsinized and the number of cells was counted. Each treatment was performed in duplicate, and each experiment was repeated thrice.

(b) Cell viability. Cell viability was determined with a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) assay. Briefly, 3,000 exponentially growing cells were seeded per well in 96-well plates and treated with 10 µg/mL protective antigen alone, or 10 µg/mL protective antigen plus 0 to 50 µg/mL lethal factor, or DMSO or U0126 (0–50 µmol/L) for 48 or 72 h. Twenty microliters of MTS reagent plus 100 µL of fresh culture medium were added to each well. After a 2-h incubation period, optical absorbance at 490 nm and a reference wavelength at 690 nm were measured. Results were expressed as percentage of viable cells relative to cells treated with protective antigen alone. Experiments were performed in triplicate and repeated at least thrice.

Soft agar colony formation assay. RCC cells were cultured in a two-layer agar system to prevent their attachment to the plastic surface. Cells (4×10^4) were trypsinized to single-cell suspensions, resuspended in 0.4% agar (Sigma), and added to a preset 1% bottom agar layer in six-well plates. The top agar cell layers were covered with culture medium containing 10% FBS and either LeTx (1 µg/mL protective antigen plus 0.5 µg/mL lethal factor), E687C (1 µg/mL protective antigen plus 0.5 µg/mL E687C), U0126 (10 µmol/L), or 0.1% DMSO or were left untreated. The top medium containing inhibitors was changed twice per week. Cells were incubated in 5% CO₂ at 37°C for 14 days, and colonies were stained with 0.005% crystal violet in methanol. The colonies with a diameter exceeding 100 µm were counted on micrographs using ImageJ v1.37v software. Experiments were performed in triplicate and repeated thrice.

Western blot analysis. Cells were cultured in 10-cm dishes as described above and harvested after 72 h of treatment. Protein was extracted from cell pellets with a lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, and 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate in the presence of protease inhibitor cocktail; Roche Diagnostics]. Samples containing equal amounts of protein (30 µg) were separated by electrophoresis on 10% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. After blocking with TTBS (TBS + 0.1% Tween 20) containing 5% nonfat milk, the membranes were incubated with mouse monoclonal antibody against phosphorylated

p44/42 MAPK (Cell Signaling), rabbit polyclonal antibody against phosphorylated SAPK/JNK (Cell Signaling), rabbit monoclonal antibody against phosphorylated p38 MAPK (Cell Signaling), rabbit polyclonal antibodies against p44/42 MAPK, SAPK/JNK (Cell Signaling), p38 MAPK (Santa Cruz), and mouse monoclonal antibody against β-actin (Abcam) at 4°C overnight. After washing with TTBS, the membranes were incubated with corresponding secondary antibodies (Santa Cruz) that were conjugated with horseradish peroxidase. Rabbit polyclonal MKK1 (Upstate), MKK3 (Santa Cruz), and rabbit monoclonal MKK6 (Epitomics) antibodies were used to detect the cleavage of MKKs by LeTx. Immunoreactive bands were visualized by autoradiography after incubation with enhanced chemiluminescence reagents (Amersham Biosciences).

Tumor implantation and growth in RCC xenograft model. All animal studies were in compliance with VARI Institutional Animal Care and Use Committee policies. Six-week-old female BALB/c nu/nu nude mice (Charles River) were given s.c. injections of 2×10^6 ACHN or A-498 cells in the right flank. Tumor size was measured thrice per week using digital calipers (Mitutoyo) that have an accuracy of ± 0.02 mm, and tumor volume was calculated as length \times width \times height \times 0.5. Tumor growth ratio is

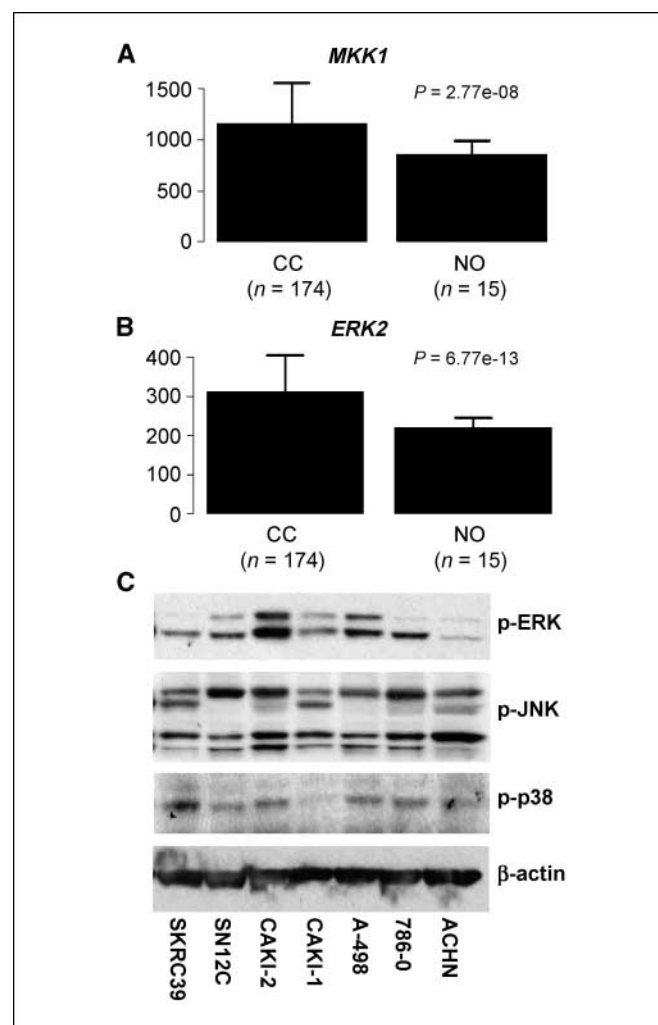


Figure 1. Overexpression of MKK1 and ERK2 mRNA in human ccRCC. Overexpression of MKK1 (A) and ERK2 (B) in 174 human clear cell RCC samples (CC) and 15 normal controls (NO) is shown by Affymetrix microarray analysis. Columns, average gene expression level of each group; bars, SD. *P* values are also shown. C, constitutive phosphorylation of MAPKs in RCC cell lines that were serum-starved for 48 h, followed by Western blots as described in Materials and Methods.

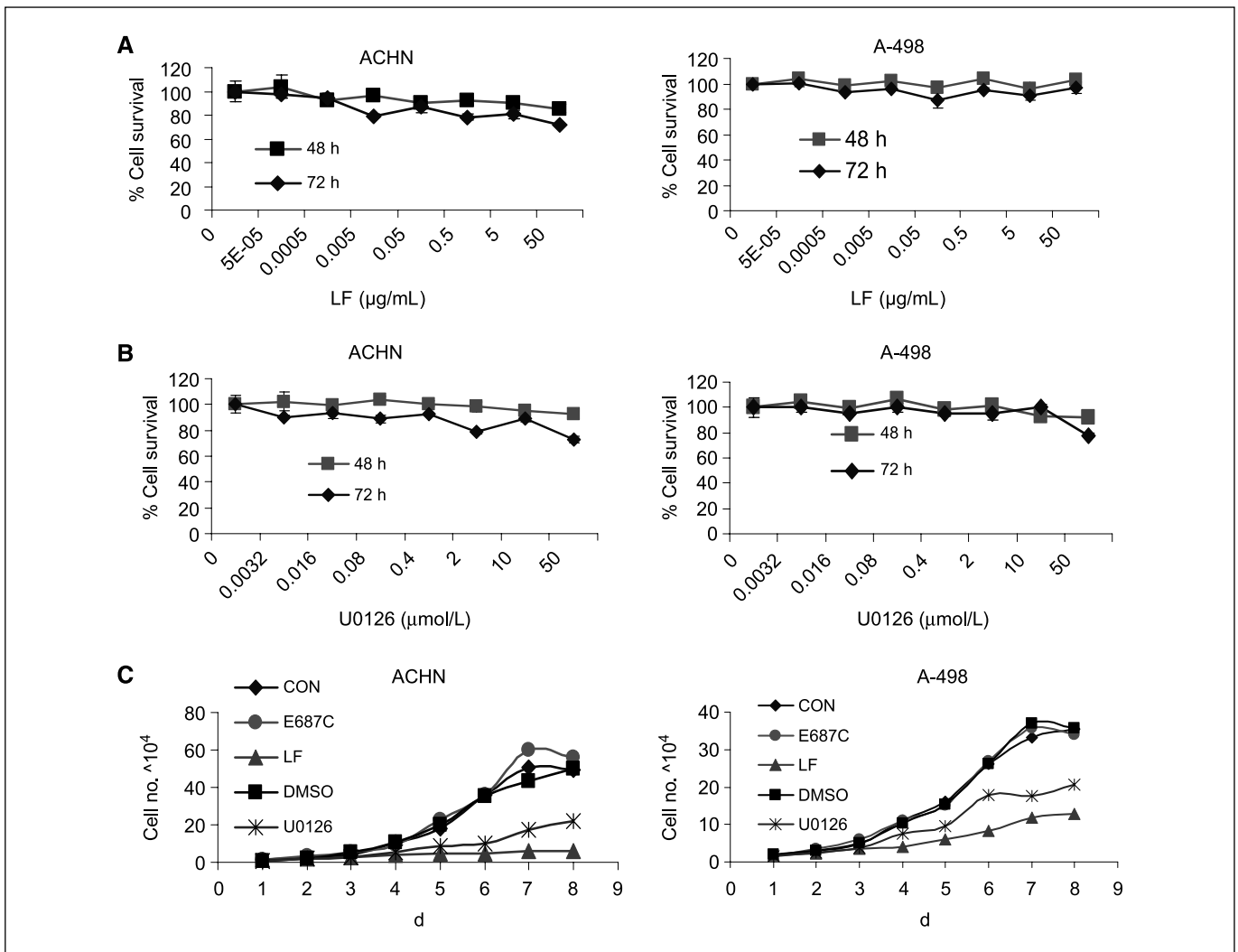


Figure 2. The effect of LeTx on proliferation of RCC cells. Viability of ACHN cells and A-498 cells after treatment with (A) LeTx [10 µg/mL protective antigen (PA) plus 0–50 µg/mL lethal factor (LF)] or (B) U0126 (0–50 µmol/L) was determined by MTS assay. Cells were treated for 48 or 72 h at the indicated concentration of LeTx or U0126. C, growth curve of ACHN and A-498 cells treated with LeTx (1 µg/mL protective antigen plus 0.5 µg/mL lethal factor) or U0126 (10 µmol/L).

presented as mean ± SD and normalized to the initial volume when treatment began. When tumors had grown to an average volume of 100 or 300 mm³ for ACHN or A-498 xenografts, respectively, tumor-bearing mice were separated into three groups of 10 animals. For ACHN tumors, all groups received i.p. injections of LeTx every other day with 2.5 standard doses for one group and two standard doses for the other (1 standard dose = 10 µg protective antigen plus 2 µg lethal factor) for a total of eight injections per group; the third group received the HBSS only (vehicle control group). For A-498 tumors, two groups received i.v. injections of LeTx thrice per week with two and one standard dose for each group for a total of five injections per group and the third group received HBSS as a control. Mice were euthanized at the end of the treatment period. Plasma samples were collected and stored at -80°C for further studies. Tumors were removed, cleaned from adjacent tissues, fixed in 4% polyformaldehyde, and paraffin-embedded, and then 4-µm-thick sections were prepared. Some sections were stained with H&E, and the others were used for subsequent immunohistochemical analysis.

Immunohistochemistry. Sections from the formalin-fixed, paraffin-embedded tumor tissues were cut to 4 µm and deparaffinized in xylene, followed by treatment with a graded series of ethanol solutions and rehydration in PBS. Immunohistochemistry staining for p-ERK (Ventana),

p-JNK (Cell Signaling), and p-p38 MAPK (Cell Signaling) were performed using optimized standard protocols on a Ventana Discovery XT staining system (Ventana). Horseradish peroxidase-conjugated UltraMap anti-mouse IgG or UltraMap anti-rabbit IgG (Ventana) were used with DAB to develop color. The proliferative index of tumor sections was determined by staining with a rabbit polyclonal proliferating cell nuclear antigen (PCNA) antibody (Abcam). To measure the effect of LeTx treatment on microvascular density, tumor sections were stained for endothelial cells using a rat monoclonal anti-mouse CD34 antibody (MEC 14.7, Abcam). Briefly, the slides were deparaffinized and rehydrated as described above. For CD34 staining, the slides were incubated with citrate buffer (pH 6.0; Zymed) at 95°C for 30 min to expose the antigen. Next, sections were immersed in 0.3% hydrogen peroxide to block endogenous peroxidase activity and then incubated in 5% goat or rabbit serum. The sections were then incubated with PCNA or CD34 antibodies overnight at 4°C in primary antibody diluting buffer (Biomed). After washing with TTBS, sections were incubated with biotinylated secondary antibodies (Vector). After washing with TTBS, sections were incubated with Vectastain ABC reagent (Vector). The immune complex was visualized using DAB substrate solution (Vector). To determine the apoptotic index, TUNEL staining was also performed on tumor sections according to the protocol of the manufacturer (Roche Diagnostics).

For PCNA and TUNEL staining, pictures of five fields (0.09 mm² each) per slide were taken using a Nuance multispectral imaging system (CRI) at 400× magnification and multispectral acquisition software. The images were processed by Nuance image processing software 1.6.8 to measure the spectral absorbance curve of each of the stains and then were unmixed. The percentage of positive staining (DAB) was then quantified using Imagine_0.16 software (developed by our laboratory) and expressed as the percentage of positive pixels to total pixels of the analyzed area. For CD34 staining, pictures of five fields per slide were taken at 200× magnification. Microvessel density was determined in a blinded manner by counting using Imagine_0.16, for each tumor, the total number of vessels in five fields (0.36 mm² each) showing the highest vascular density (33). For other antibodies, the whole section was reviewed and scored semiquantitatively using a visual grading system based on the intensity of staining (–, negative; +, weak; ++, modest; +++, strong), and the percentage of staining (0, 1–25%, 26–50%, 51–75%, >75%) according to the intensity of chromogen deposition in the majority of cancer cells as evaluated independently by two observers (34).

Statistical analysis. All values are expressed as mean ± SD. Values were compared using Student's *t* test. *P* < 0.05 was considered significant.

Results

MKK and MAPK are overexpressed in human ccRCC. To check the status of MKK signaling pathways in RCC, we first

checked the expression levels of *MKKs* and *MAPKs* in 174 human clear cell RCC samples by Affymetrix microarray analysis. We found that *MKK1* and *ERK2* were significantly elevated in human ccRCC samples compared with normal controls (*P* < 0.001; Fig. 1A and B). We also examined the basal activation of MAPKs in RCC cell lines by Western blotting. Constitutive activation of ERK, JNK, and p38 MAPK were observed in RCC cell lines (Fig. 1C).

LeTx showed modest effect on proliferation of RCC cells *in vitro*. Because signaling through MKK is thought to play an essential role in the proliferation of cancer cells, we evaluated the effect of inhibition of MKK, using either LeTx or U0126 (a specific MKK1/2 inhibitor), on the proliferation of RCC cells *in vitro*. Previous studies about how lethal factor enters the cell and inhibits MKKs have shown that protective antigen forms heptamer while binding to lethal factor, so the ideal working molecule ratio of protective antigen and lethal factor is 5:1 to 7:1. Therefore, we used 10 μg/mL protective antigen (enough for most lethal factor doses) plus 0 to 50 μg/mL lethal factor in proliferation assay (MTS). Proliferation of ACHN cells was inhibited by 20% to 30% by LeTx relative to controls, but little effect was observed on A-498 cells after 72 h of LeTx treatment (Fig. 2A). The effect of LeTx on other RCC cell lines (described in Materials and Methods) was also

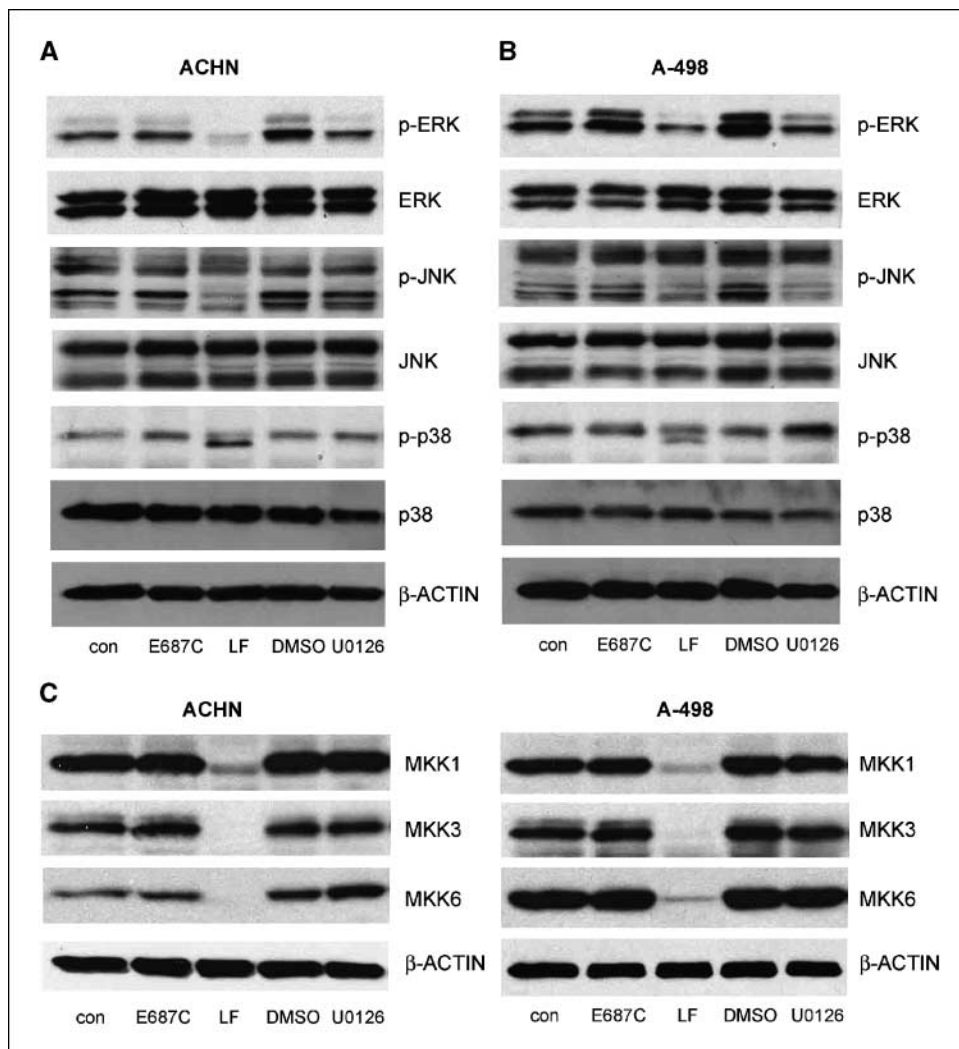


Figure 3. Inhibition of phosphorylation of ERK, JNK, and p38 MAPKs by LeTx in RCC cells. LeTx inhibited phosphorylation of MAPKs in ACHN (A) and A-498 (B) cells. Cells were treated with either LeTx (1 μg/mL protective antigen plus 0.5 μg/mL lethal factor), E687C (1 μg/mL protective antigen plus 0.5 μg/mL E687C), U0126 (10 μmol/L from 10 mmol/L stock solution in DMSO), or DMSO (control), or were left untreated for 72 h. Then cells were harvested, and Western blotting was done as described in Materials and Methods. C, cleavage of MKK1, MKK3, and MKK6 by LeTx in ACHN and A-498 cells.

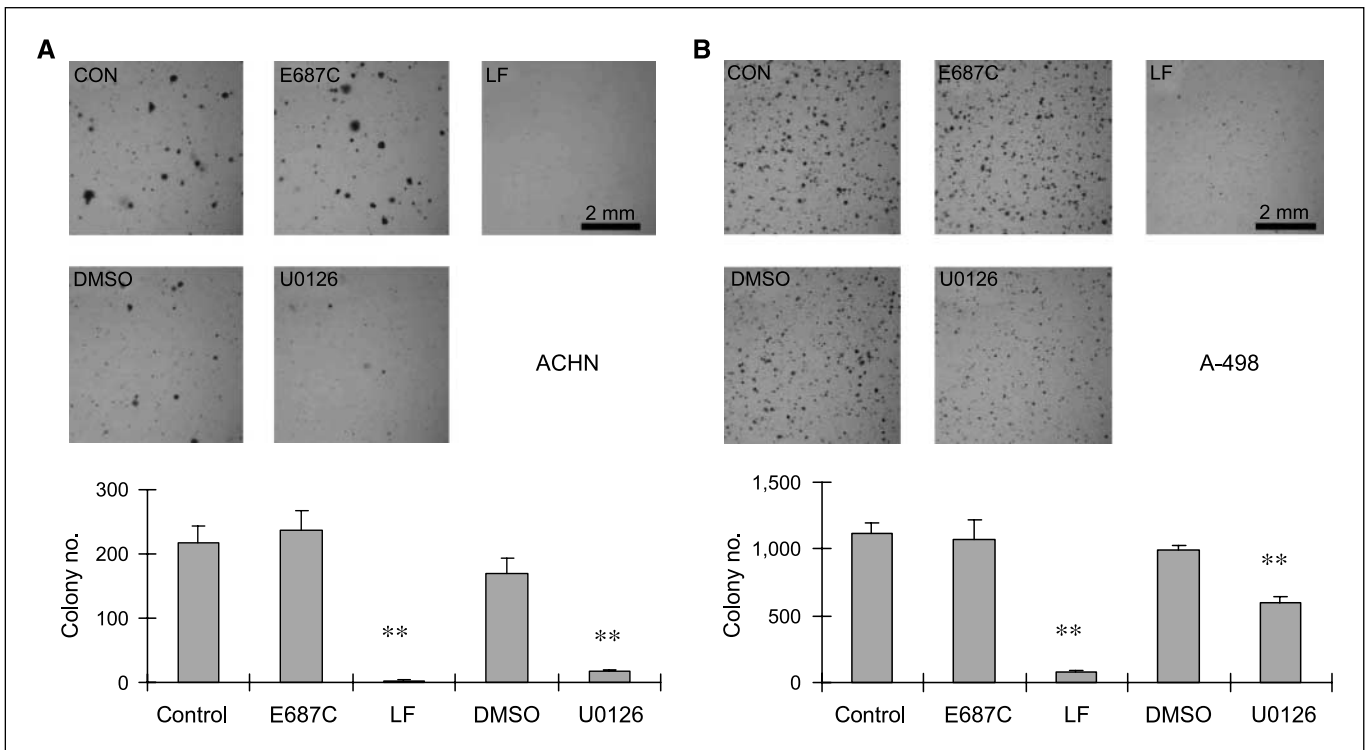


Figure 4. The effect of LeTx on anchorage-independent colony formation of RCC cells. Colony formation of ACHN (A) and A-498 (B) cells in soft agar was inhibited by LeTx (1 µg/mL protective antigen plus 0.5 µg/mL lethal factor) and U0126 (10 µmol/L). **, *P* < 0.01.

tested; the proliferation of only 4 of 10 other RCC cell lines could be inhibited by 20% to 30% after 72 h of LeTx exposure (Supplementary Fig. S1A). Exposure to U0126 gave similar results (Fig. 2B and Supplementary Fig. S1B). The growth curves of ACHN and A-498 cells treated with LeTx or U0126 were shown in Fig. 2C. LeTx and U0126 showed robust inhibition on the proliferation of ACHN and A-498 cells after a longer period of treatment (8 days) relative to E687C and DMSO controls. We also got similar result with MTS assay after the cells were treated with LeTx for 7 days (Supplementary Fig. S1C).

LeTx inhibited phosphorylation of ERK, JNK, and p38 MAPKs in RCC cells *in vitro*. We then tested whether the inhibitory effect on cell proliferation by LeTx was associated with the reduced phosphorylation levels of MAPKs in RCC cells. The major function of MKKs is to phosphorylate and activate downstream MAPKs, and cleavage of MKKs by LeTx will result in loss of this function. Measuring the phosphorylation levels of MAPKs could therefore reflect the inhibition of MKKs. There are three major MAPKs: ERK, JNK, and p38 MAPK (p38). We used 0.5 µg/mL lethal factor plus 1 µg/mL protective antigen and 10 µmol/L U0126, which was shown as the most effective dose in proliferation assay (see Fig. 2 and Supplementary Fig. S1) to treat the cells. Moreover, 10 µmol/L U0126 has also been shown to be sufficient to inhibit ERK phosphorylation in melanoma cells (28). After treatment with LeTx for 72 h, phosphorylation of ERK, JNK, and p38 kinases in ACHN and A-498 cells was inhibited, relative to E687C and untreated controls (Fig. 3A and B). U0126 showed similar result on phosphorylated ERK. The cleavage of MKK1, MKK3, and MKK6 was also confirmed by Western blotting using an NH₂ terminal-specific antibody. All MKKs were effectively cleaved by LeTx treatment (Fig. 3C). To rule out the

stimulating effect of multiple growth factors existing in complete medium, we also checked the effect of LeTx on activation of MAPKs under serum-starved condition. Similar results were found (Supplementary Fig. S2). LeTx could also inhibit the constitutively activated MAPKs.

LeTx inhibited anchorage-independent growth of RCC cells. Anchorage-independent growth is a characteristic of transformed cells. Intrinsic anchorage-independent growth activity *in vitro* closely reflects the tumorigenicity of epithelial cells. Loss of anchorage dependence leads to reversible growth arrest of fibroblasts and apoptosis of epithelial and endothelial cells (35, 36). After 14 days of exposure, LeTx inhibited the anchorage-independent colony formation of ACHN and A-498 cells by 90% at 0.5 µg/mL (Fig. 4). Similar results were obtained with U0126 (10 µmol/L).

LeTx inhibited RCC tumor growth and neovascularization *in vivo*. RCC xenografts are slowly growing tumors. We tested the effect of LeTx on established tumors after s.c. inoculation of the cancer cells in the right flank of nude mice. Treatment began when the average tumor volume reached 100 or 300 mm³ for ACHN or A-498 xenografts, respectively. I.p. injection of LeTx was performed in the ACHN xenograft model. The tumor growth was inhibited by 90% using 2 and 2.5 standard doses of LeTx (referred as 2× and 2.5× LeTx in Supplementary Fig. S3). For A-498 xenograft model, i.v. injection was performed. The tumor growth was inhibited by one standard dose of LeTx (referred as 1× LeTx), and tumor regression was observed with two standard doses of LeTx (referred as 2× LeTx in Fig. 5) treatment. The *in vivo* dose was based on the result of previous studies that have shown using 10 µg protective antigen plus 2 µg lethal factor per mouse, effective but not lethal, as the standard dose

for *in vivo* experiment (27, 28). Another study done by Abi-Habib et al. also showed that 5:1 ratio of protective antigen to lethal factor produced significant tumor growth inhibition on melanoma (30). An increased necrotic area was observed in LeTx-treated sections compared with vehicle controls after H&E staining (note the decreased viable rim in Fig. 5B and Supplementary Fig. S3B).

To confirm that the growth inhibition effect was correlated with MKK signaling inhibition, we performed phosphorylated MAPK staining in the tumor sections. Modest or strong staining of phosphorylated ERK, phosphorylated JNK, and phosphorylated p38 were found in viable tumor sections of HBSS-treated controls, whereas only weak or modest staining of the above antigens were found in viable tumor sections of LeTx-treated tumors, especially in the cytoplasmic compartment (Fig. 5B and Supplementary Fig. S3B).

We used PCNA staining as a marker for proliferating cells in tumor sections. For the A-498 xenografts, the number of PCNA-positive cells (as shown by proliferation index) decreased slightly after LeTx treatment when compared with the controls (Fig. 5C). Extensive DNA fragmentation may occur in late stages of necrosis (37, 38), which would also be positive in TUNEL staining. The large necrotic area after LeTx treatment was also positively stained by the TUNEL method. Therefore, we only count TUNEL-positive cells in the viable tumor section (rim) to calculate the percentage of apoptosis induced by LeTx *in vivo*. In both ACHN and A-498 xenografts, no increase of apoptotic cells (as shown by apoptosis index) was found after LeTx treatment (Fig. 5C and Supplementary Fig. S3C). But an increased area of necrotic cells (also TUNEL positive) was observed in the tumor sections treated by LeTx.

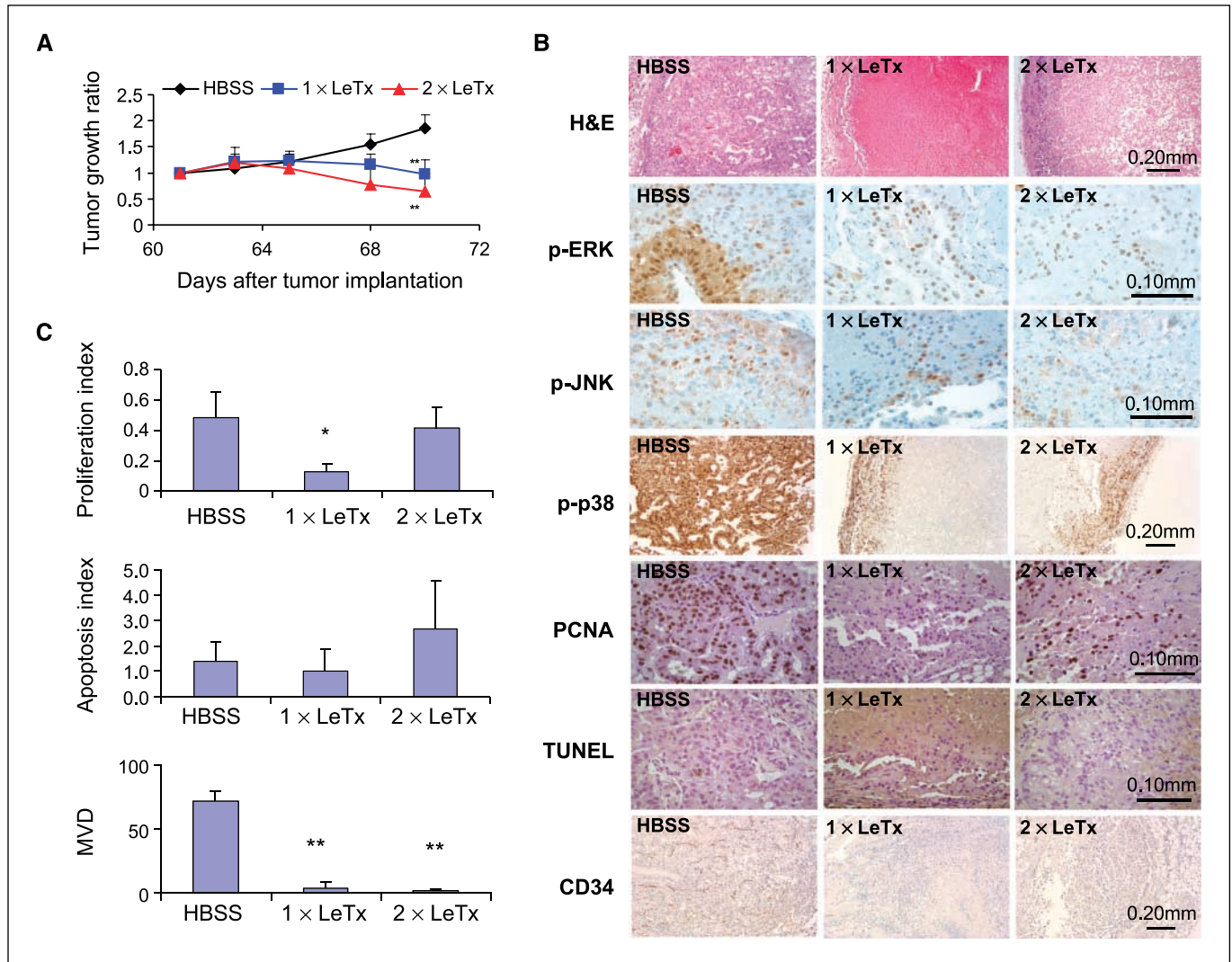


Figure 5. The effects of LeTx upon growth of RCC xenografts and histologic analyses of tumors derived from LeTx-treated mice. A, tumor growth curve was plotted for A-498 xenograft after treatment with LeTx ($n = 10$ mice per group). I.v. injection of one standard dose of LeTx ($1 \times \text{LeTx} = 10 \mu\text{g}$ protective antigen + $2 \mu\text{g}$ lethal factor) induced growth inhibition; injection of two standard doses of LeTx ($2 \times \text{LeTx} = 20 \mu\text{g}$ protective antigen + $4 \mu\text{g}$ lethal factor) even induced tumor regression relative to HBSS control. Points, mean of tumor growth ratio normalized to the initial volume when treatment began; bars, SD. B, H&E staining and immunohistochemical staining for phosphorylated MAPKs, PCNA, TUNEL, and CD34 in A-498 xenograft tumors treated by LeTx. A larger necrotic area was found with H&E staining in LeTx-treated tumor sections as shown by a thinner proliferating rim. Activation of MAPKs was also inhibited by LeTx treatment *in vivo*, especially in the cytoplasmic compartment. C, quantification of a proliferation index and an apoptosis index in the viable rim of A-498 xenograft tumors and quantification of microvessel density of A-498 xenograft tumors treated by LeTx were also shown. *, $P < 0.05$; **, $P < 0.01$.

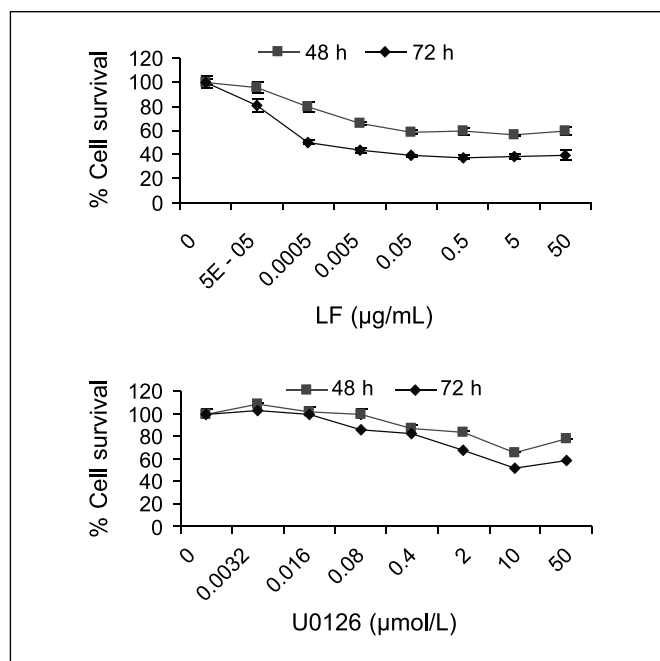


Figure 6. Effect of LeTx and U0126 on proliferation of HUVEC cells. Viability of HUVEC cells after treatment with LeTx (10 $\mu\text{g/mL}$ protective antigen plus 0–50 $\mu\text{g/mL}$ lethal factor) or U0126 (0–50 $\mu\text{mol/L}$) was determined by MTS assay. Cells were treated for 48 or 72 h at the indicated concentration of LeTx or U0126.

We observed the extensively necrotic areas in LeTx-treated tumor sections. Because LeTx only modestly inhibited tumor cells proliferation but did not induce apoptosis, we speculate that the antitumor effect may be mainly caused by inhibition of tumor angiogenesis. Therefore, we used CD34, a vascular endothelial cell biomarker, to stain the tumor sections and counted the microvessel density. The microvessel density was significantly decreased after LeTx treatment when compared with HBSS-treated controls ($P < 0.001$; Fig. 5C). We also examined the direct effect of LeTx on endothelial cell proliferation *in vitro*; LeTx showed more profound inhibition on endothelial cell proliferation than U0126 (Fig. 6).

Discussion

The role of RAS/RAF/MKK/ERK signaling in tumorigenesis and metastasis has been well established. Further studies have suggested that other MKK pathways may also play a role in angiogenesis (39–42). MKK inhibition is an attractive anticancer strategy, as it has the potential to block inappropriate signal transduction regardless of the upstream position of the oncogenic aberration. As a multiple MKK inhibitor, LeTx is different from most small-molecule MKK inhibitors, which only block MKK1/MKK2 activity. LeTx acts through the cell surface receptor, cleaving and inactivating multiple MKKs.

Our cellular study showed that LeTx could inhibit phosphorylation of ERK, JNK, and p38 MAPKs in RCC cells under both normal and serum-starved conditions, but we also observed an extra band of phosphorylated p38 MAPK (Fig. 3A and B), which might be differentially phosphorylated isoforms of p38 MAPK. Four isoforms of p38 kinase (α , β , γ , and δ) have been defined in mammalian cells. All four isoforms can be activated by MKK6; p38 α and p38 γ

are also activated by MKK3 (43, 44). We reconfirmed the cleavage of MKK3 and MKK6 kinases using their NH_2 -terminal antibodies and found that both MKKs were cleaved by LeTx treatment. Decreased phosphorylation of some isoforms of p38 kinase may be due to the inhibition of certain phosphorylation sites.

The proliferation of RCC cells was only inhibited modestly after treatment with LeTx for 72 h *in vitro*, but if the cells were treated for a longer duration (see Fig. 2C and Supplementary Fig. S1C); robust inhibition of proliferation was observed relative to controls. The doubling time of RCC cells *in vitro* is ~ 48 h. LeTx may only slow down cell cycle progression rather than killing the cells, so it took several cell cycles to reflect this effect, i.e., decrease in cell number. No significant effect on cell cycle distribution and apoptosis was observed from LeTx treatment both for 72 h and 7 days (Supplementary Figs. S4 and S5). U0126 showed similar results as LeTx, but was less effective, which shows that inhibition of multiple MKK signaling is of more value to RCC cell proliferation than a single MKK/ERK pathway.

In vivo, we observed similar result. Proliferation of A-498 cells, but not ACHN cells (as shown by PCNA staining), was partially inhibited by LeTx treatment without induction of apoptosis (as shown by TUNEL staining). The activation of MAPKs was also inhibited, especially in the cytoplasmic compartment, leaving a smaller portion of activated MAPKs in the nucleus after LeTx treatment. Despite the weak antiproliferation effect of LeTx on RCC cells, profound tumor growth inhibition was observed after LeTx treatment in RCC xenograft models. Extensive necrosis was also observed in the center of tumors after four to five treatments with LeTx. Because we observed weak or modest effects of LeTx on tumor cell proliferation and apoptosis, the antitumor effect *in vivo* is most likely caused by the antiangiogenic effect. The extensive tumor necrosis may be a result of the interruption of tumor vasculature.

Previous studies have shown that activation of MKK signal transduction pathways in endothelial cells is required for angiogenesis. MKK activity regulates vascular endothelial growth factor (VEGF) expression at the transcriptional and posttranscriptional levels (45). The role of ERK activation in angiogenic growth factor signaling has been well established (46). ERK activation is probably required for the growth factor–induced secretion of angiogenic factors from tumor cells (47). Here, we examined the direct effect of inhibition of MAPKs signaling with LeTx on endothelial cell proliferation. LeTx showed robust inhibition of endothelial cells, which is consistent with the findings of others (29, 39, 48).

Frequent VHL mutation, which leads to the overexpression of VEGF through HIF signaling, was found in the majority of clear cell RCC patients and may contribute to angiogenesis in tumors. Because growth factors often act through autocrine and/or paracrine routes, we also investigated the production of VEGF by RCC cells and/or host cells both *in vitro* and *in vivo*. But the secretion of VEGF by A-498 and ACHN cells both *in vitro* and *in vivo* was not inhibited by LeTx treatment, whereas the production of VEGF by mouse stromal cells *in vivo* was slightly inhibited by LeTx treatment (Supplementary Fig. S6). The fact that LeTx failed to inhibit the secretion of VEGF by RCC cells suggests that LeTx may act directly on endothelial cells *in vivo*. These data indicate that the antitumor efficacy of LeTx may be primarily attributable to angiogenesis inhibition resulting from the inhibition of MKK signaling in endothelial cells. More studies are needed to validate this hypothesis.

In conclusion, in this study we show that LeTx exhibits significant antitumor activity in RCC xenograft models via an antiangiogenic effect. Previous studies have shown that LeTx induced dramatic apoptosis and cell death in melanoma cells and indicated that LeTx may be useful as a therapeutic agent in clinical trials of melanoma. The potent antiangiogenic effects of LeTx that we showed here suggest that its use in clinical settings may not restrict in melanoma. Given the highly vascularized nature of RCC, LeTx warrants further study as a therapeutic agent for RCC.

Acknowledgments

Received 9/14/2007; revised 10/30/2007; accepted 10/31/2007.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
- Motzer RJ, Bander NH, Nanus DM. Renal-cell carcinoma. *N Engl J Med* 1996;335:865–75.
- Goodman VL, Rock EP, Dagher R, et al. Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. *Clin Cancer Res* 2007;13:1367–73.
- Rock EP, Goodman V, Jiang JX, et al. Food and Drug Administration drug approval summary: sunitinib malate for the treatment of gastrointestinal stromal tumor and advanced renal cell carcinoma. *Oncologist* 2007;12:107–13.
- Kane RC, Farrell AT, Saber H, et al. Sorafenib for the treatment of advanced renal cell carcinoma. *Clin Cancer Res* 2006;12:7271–8.
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001;410:37–40.
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911–2.
- Dunn KL, Espino PS, Drobcic B, He S, Davie JR. The Ras-MAPK transduction pathway, cancer and chromatin remodeling. *Biochem Cell Biol* 2005;83:1–14.
- Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–54.
- Jeffers M, Fiscella M, Webb CP, Anver M, Koochekpour S, Vande Woude GF. The mutationally activated Met receptor mediates motility and metastasis. *Proc Natl Acad Sci U S A* 1998;95:14417–22.
- Webb CP, Taylor GA, Jeffers M, et al. Evidence for a role of Met-HGF/SF revised Ras-mediated tumorigenesis/metastasis. *Oncogene* 1998;17:2019–25.
- Ward Y, Wang W, Woodhouse E, Linnoila I, Liotta L, Kelly K. Signal pathways which promote invasion and metastasis: critical and distinct contributions of extracellular signal-regulated kinase and Ral-specific guanine exchange factor pathways. *Mol Cell Biol* 2001;21:5958–69.
- Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest* 1997;99:1478–83.
- Mandell JW, Hussaini IM, Zecevic M, Weber MJ, VandenBerg SR. *In situ* visualization of intratumor growth factor signaling: immunohistochemical localization of activated ERK/MAP kinase in glial neoplasms. *Am J Pathol* 1998;153:1411–23.
- Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 1999;18:813–22.
- Salh B, Marotta A, Matthewson C, et al. Investigation of the Mek-MAP kinase-Rsk pathway in human breast cancer. *Anticancer Res* 1999;19:731–40.
- Giroux S, Tremblay M, Bernard D, et al. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. *Curr Biol* 1999;9:369–72.
- Berra E, Milanini J, Richard DE, et al. Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem Pharmacol* 2000;60:1171–8.
- Dong G, Chen Z, Li ZY, Yeh NT, Bancroft CC, Van Waes C. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. *Cancer Res* 2001;61:5911–8.
- Elceiri BP, Klemke R, Stromblad S, Cheresch DA. Integrin $\alpha v \beta 3$ requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *J Cell Biol* 1998;140:1255–63.
- Depeille P, Young JJ, Boguslawski EA, et al. Anthrax lethal toxin inhibits growth of and vascular endothelial growth factor release from endothelial cells expressing the human herpes virus 8 viral g protein coupled receptor. *Clin Cancer Res* 2007;13:5926–34.
- Oka H, Chatani Y, Hoshino R, et al. Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res* 1995;55:4182–7.
- Duesbery NS, Webb CP, Leppla SH, et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* 1998;280:734–7.
- Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFN γ -induced release of NO and TNF α . *FEBS Lett* 1999;462:199–204.
- Vitale G, Pellizzari R, Recchi C, Napolitano G, Mock M, Montecucco C. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem Biophys Res Commun* 1998;248:706–11.
- Liang X, Young JJ, Boone SA, Waugh DS, Duesbery NS. Involvement of domain II in toxicity of anthrax lethal factor. *J Biol Chem* 2004;279:52473–8.
- Duesbery NS, Resau J, Webb CP, et al. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. *Proc Natl Acad Sci U S A* 2001;98:4089–94.
- Koo HM, VanBrocklin M, McWilliams MJ, Leppla SH, Duesbery NS, Woude GF. Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. *Proc Natl Acad Sci U S A* 2002;99:3052–7.
- Abi-Habib RJ, Urieto JO, Liu S, Leppla SH, Duesbery NS, Frankel AE. BRAF status and mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin. *Mol Cancer Ther* 2005;4:1303–10.
- Abi-Habib RJ, Singh R, Leppla SH, et al. Systemic anthrax lethal toxin therapy produces regressions of subcutaneous human melanoma tumors in athymic nude mice. *Clin Cancer Res* 2006;12:7437–43.
- Park S, Leppla SH. Optimized production and purification of *Bacillus anthracis* lethal factor. *Protein Expr Purif* 2000;18:293–302.
- Furge KA, Chen J, Koeman J, et al. Detection of DNA copy number changes and oncogenic signaling abnormalities from gene expression data reveals MYC activation in high-grade papillary renal cell carcinoma. *Cancer Res* 2007;67:3171–6.
- Murphy DA, Makonnen S, Lassoued W, Feldman MD, Carter C, Lee WM. Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). *Am J Pathol* 2006;169:1875–85.
- Nomura T, Huang WC, Zhou HE, et al. $\beta 2$ -Microglobulin promotes the growth of human renal cell carcinoma through the activation of the protein kinase A, cyclic AMP-responsive element-binding protein, and vascular endothelial growth factor axis. *Clin Cancer Res* 2006;12:7294–305.
- Aplin AE, Howe AK, Juliano RL. Cell adhesion molecules, signal transduction and cell growth. *Curr Opin Cell Biol* 1999;11:737–44.
- Frisch SM, Ruoslahti E. Integrins and anoikis. *Curr Opin Cell Biol* 1997;9:701–6.
- Gold R, Schmed M, Giegerich G, et al. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab Invest* 1994;71:219–25.
- Collins RJ, Harmon BV, Gobe GC, Kerr JF. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int J Radiat Biol* 1992;61:451–3.
- Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* 1997;15:2169–77.
- Clauss M, Sunderkotter C, Sveinbjornsson B, et al. A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood* 2001;97:1321–9.
- Sodhi A, Montaner S, Patel V, et al. The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1 α . *Cancer Res* 2000;60:4873–80.
- Vasilevskaya I, O'Dwyer PJ. Role of Jun and Jun kinase in resistance of cancer cells to therapy. *Drug Resist Update* 2003;6:147–56.
- Qi M, Elion EA. MAP kinase pathways. *J Cell Sci* 2005;118:3569–72.
- Enslin H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the map kinase kinases MKK3 and MKK6. *J Biol Chem* 1998;273:1741–8.
- Depeille PE, Ding Y, Bromberg-White JL, Duesbery NS. MKK signaling and vascularization. *Oncogene* 2007;26:1290–6.
- Milanini J, Vinals F, Pouyssegur J, Pages G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem* 1998;273:18165–72.
- Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997;151:1523–30.
- Kirby JE. Anthrax lethal toxin induces human endothelial cell apoptosis. *Infect Immun* 2004;72:430–9.