

Mitogen-activated protein kinase kinase signaling promotes growth and vascularization of fibrosarcoma

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Abstract

We hypothesized that signaling through multiple mitogen-activated protein kinase (MAPK) kinase (MKK) pathways is essential for the growth and vascularization of soft-tissue sarcomas, which are malignant tumors derived from mesenchymal tissues. We tested this using HT-1080, NCI, and Shac fibrosarcoma-derived cell lines and anthrax lethal toxin (LeTx), a bacterial toxin that inactivates MKKs. Western blots confirmed that LeTx treatment reduced the levels of phosphorylated extracellular signal-regulated kinase and p38 MAPK *in vitro*. Although short treatments with LeTx only modestly affected cell proliferation, sustained treatment markedly reduced cell numbers. LeTx also substantially inhibited the extracellular release of angioproliferative factors including vascular endothelial growth factor, interleukin-8, and basic fibroblast growth factor. Similar results were obtained with cell lines derived from malignant fibrous histiocytomas, leiomyosarcomas, and liposarcomas. *In vivo*, LeTx decreased MAPK activity and blocked fibrosarcoma growth. Growth inhibition correlated with decreased cellular proliferation and extensive necrosis, and it was accompanied by a decrease in tumor mean vessel density as well as a reduction in serum expression of angioproliferative cytokines. Vital imaging using high-resolution ultrasound enhanced with contrast

microbubbles revealed that the effects of LeTx on tumor perfusion were remarkably rapid (<24 h) and resulted in a marked reduction of perfusion within the tumor but not in nontumor tissues. These results are consistent with our initial hypothesis and lead us to propose that MKK inhibition by LeTx is a broadly effective strategy for targeting neovascularization in fibrosarcomas and other similar proliferative lesions. [Mol Cancer Ther 2008;7(3):648–58]

Introduction

Mitogen-activated protein kinase (MAPK) kinase (MKK) signal transduction pathways are critical for many aspects of normal cell function including cell cycle progression and differentiation (1). In addition, activated MAPK or elevated MAPK expression has been detected in a variety of human tumor, where it is believed they promote tumor growth and metastasis (2). These observations have spurred the development of drugs that target the MKK pathways as potential cancer therapeutics (3).

Most of what is known of MKK signaling in neoplasia is based on research into carcinomas. By contrast, little is known about the role of MKK signaling pathways in soft-tissue sarcoma. Sarcomas are tumors derived from mesenchymal tissues, and they represent ~1% of adult and 16% of pediatric malignancies. Although elevated levels of active MAPK have been detected *in vitro* in cell lines derived from fibrosarcoma (4–6), rhabdomyosarcoma (7), osteosarcoma (8), and Kaposi's sarcoma (9, 10), little is known of their activities *in vivo*. Based on our earlier work with transformed fibroblasts (11), we hypothesized that signaling through multiple MKK pathways is essential for the growth and vascularization of xenograft sarcomas.

To test this hypothesis, we have treated fibrosarcoma-derived cell lines and xenografts with anthrax lethal toxin (LeTx). LeTx is a binary toxin consisting of protective antigen and lethal factor. Protective antigen is nontoxic and facilitates entry of lethal factor into the cell. Lethal factor is a Zn²⁺-metalloprotease that cleaves and inactivates MKK1, MKK2, MKK3, MKK4, MKK6, and MKK7 but not MKK5 (12). The results of these studies indicate that although MKK signaling plays a modest role in proliferation, it substantially inhibits the extracellular release of angioproliferative factors including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) *in vitro*. *In vivo*, MKK signaling is necessary for tumor growth and vascularization. Moreover, the pattern of tumor cell death elicited by LeTx, as well as the rapid onset of vascular dysfunction in its presence, leads us to conclude that the predominant fibrosarcoma-related activity of MKK *in vivo* is proangiogenic likely through the regulation of proangiogenic cytokines such as VEGF.

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Materials and Methods

Cell Lines and Treatments

The HT-1080 human fibrosarcoma cell line, the GCT cell line (human fibrous histiocytoma), the SK-LMS and SK-UT-1 cell lines (human leiomyosarcoma), the SW-872 cell line (human liposarcoma), and the A-673 and A-204 cell lines (human rhabdomyosarcoma) were all obtained from the American Tissue Culture Collection. The NCI and Shac human fibrosarcoma cell lines were a gift from Dr. J. McCormick (Michigan State University). HT-1080, NCI, Shac, SK-LMS, and SK-UT-1 were cultured in Eagle's MEM, modified by addition of L-aspartic acid (0.2 mmol/L), L-serine (0.2 mmol/L), and pyruvate (1 mmol/L). A-673 cells were cultured in DMEM. A-204 and GCT cells were cultured in McCoy's 5a medium, and SW-872 were cultured in Leibovitz L-15 medium supplemented with 2 mmol/L L-glutamine. All of the culture media contained 10% fetal bovine serum (Invitrogen), penicillin (100 units/mL), and streptomycin (100 µg/mL; Invitrogen). All cell lines were routinely cultured at 37°C in a humidified incubator containing 5% CO₂, with the exception of the SW-872 cell line, which was cultured at 37°C in a humidified incubator without added CO₂.

Protective antigen, inactive lethal factor (E687C), and active lethal factor were purified from cultures of *Bacillus anthracis* as described (13). A 50 mmol/L stock solution of U0126 (Calbiochem), and 10 mmol/L stock solutions of nocodazole and aphidicolin (Calbiochem) in DMSO were prepared and stored in aliquots at -80°C until required.

Cell Proliferation Analysis

Cells were cultured in 96-well plates until they reached 30% to 50% confluency. Protective antigen (1 µg/mL) plus lethal factor (0.01-10,000 ng/mL) were then added to the culture medium and cells were incubated a further 24, 48, or 72 h, at which point cell viability was assayed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Each experiment was done in triplicate and was repeated at least three times.

Cell Cycle Analysis

The cell cycle profile was assessed by fluorescence-activated cell sorting using propidium iodide (PI) staining. Cells were trypsinized, washed with Versene, fixed with 70% ethanol, and stained using the Cellular DNA Flow Cytometric Analysis kit (Roche) according to the manufacturer's instructions. Cells were sorted with a FACSCalibur flow cytometer, and the percentages of cells in sub-G₁, G₀-G₁, S, and G₂-M phases of the cell cycle were measured using FACSCalibur software programs. Experiments were repeated at least three times.

Apoptosis and Necrosis Analysis

Apoptosis/necrosis was quantified by fluorescence-activated cell sorting using Annexin V and PI double staining. After overnight serum starvation, cells in 10-cm dishes were treated with either the medium alone, DMSO (0.02%), 1 µg/mL protective antigen plus 100 ng/mL E687C, 1 µg/mL protective antigen plus 100 ng/mL lethal factor, 10 µmol/L U0126, 0.05 µmol/L nocodazole, or 1 µmol/L aphidicolin

for 72 h. Then cells were trypsinized, washed with PBS, and stained using the Annexin V-FITC apoptosis detection (BD Biosciences) according to the manufacturer's instructions. Cells were sorted with a FACSCalibur flow cytometer and the percentage of cells staining positive for PI only (necrotic cells), positive for Annexin V (apoptotic cells), or negative for both (healthy cells) was measured using FACSCalibur software. Experiments were repeated at least three times.

Immunoblotting

When cells growing in 10-cm plates reached 30% to 50% confluency, they were treated with either the medium alone, DMSO (0.02%), 1 µg/mL protective antigen plus 100 ng/mL E687C, 1 µg/mL protective antigen plus 100 ng/mL lethal factor, or 10 µmol/L U0126 for 72 h. Then, the cells were lysed in RIPA buffer for SDS-PAGE and immunoblotted using antibodies raised against human phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (MAPK1/2; 1:2,000; Cell Signaling), ERK1/2 (1:1,000; Cell Signaling), c-Jun NH₂-terminal kinase (JNK; 1:1,000; Cell Signaling), phosphorylated JNK (1:1,000; Cell Signaling), p38 MAPK (1:1,000; Cell Signaling), phosphorylated p38 MAPK (1:1,000; Cell Signaling), and α-tubulin (1:5,000; Sigma). Membranes were probed with horseradish peroxidase-conjugated anti-mouse/rabbit antibodies (1:5,000) and specific proteins were visualized using X-ray films after incubation with enhanced chemiluminescence reagents (Cell Signaling; ref. 11).

VEGF and Cytokine Release

When cells growing in 10-cm plates reached 50% to 60% confluency, they were treated with either the medium alone, DMSO (0.02%), 1 µg/mL protective antigen plus 100 ng/mL E687C, 1 µg/mL protective antigen plus 100 ng/mL lethal factor, or 10 µmol/L U0126. After 24 h of treatment, fresh medium was added and the cells were incubated for a further 24 h. This conditioned medium was then filtered through a 0.22 µm membrane, mixed with 10× conditioned medium buffer (250 mmol/L HEPES, 1% bovine serum albumin, 10 mmol/L EDTA, and 1 EDTA-Free Protease Tablet for 100 mL), and stored in aliquots at -80°C until needed. At the same time, the cells were lysed by RIPA buffer and the concentration of total protein was determined by the BCA method (14).

VEGF released into the conditioned medium was measured with a human VEGF ELISA kit (Calbiochem). The same samples were also sent to Rules-Based Medicine for cytokine screening (human MAP service version 1.6). All of the results were normalized to total protein concentration.

Xenograft Studies

All experiments were done in compliance with the guiding principles of the "Guide for the Care and Use of Laboratory Animals." All procedures were approved before use by the Institutional Animal Care and Use Committee of the Van Andel Research Institute. Cells (2×10^6 - 5×10^6 in a volume of 100 µL) were injected s.c. into the dorsal flank of athymic nude mice. LeTx treatment began when tumors reached a volume of ~100 mm³. Groups of six mice were

injected every second day through the tail vein with either 100 μ L protective antigen plus E687C (2 standard doses; 1 standard dose = 10 μ g protective antigen and 2 μ g lethal factor or E687C) or LeTx (0.5, 1, and 2 standard doses). Tumor dimensions were measured three times per week using digital calipers (Mitutoyo) that have an accuracy of ± 0.02 mm. Volume was calculated by multiplying length \times width \times depth $\times 1/2$. Blood samples, taken by the retro-orbital route, were collected for analysis before cell line injection, before toxin treatment, and again at the end of the project. At the end of the experiments, the mice were euthanized, additional blood samples were collected, and the tumors were fixed in 4% neutral-buffered formalin.

Evaluation of Tumor Perfusion by Ultrasound

All ultrasound imaging was done with the VEVO 770 High-Resolution Micro-Ultrasound platform (VisualSonics). The vascular patterns of both the tumor and kidney regions were assessed noninvasively before treatment or 24 h after treatment by image enhancement with ultrasound contrast microbubbles (Vevo MicroMarker Non-Targeted Contrast Agent Kit) in conjunction with a dynamic imaging sequence.

Briefly, before each study, a fresh vial of microbubbles was reconstituted with saline to a final suspension of (2×10^9) microbubbles/mL according to the supplier's instructions. For each pretreatment or posttreatment imaging study, a baseline image sequence was first acquired using the RMV 706 probe in contrast mode at a frequency of 40 MHz, and then a dynamic sequence was acquired beginning just before microbubble injection and continuing for up to 60 s, for a total of 675 frames postinjection. For each injection, a bolus of 1×10^8 microbubbles was administered in a volume of 50 μ L via a 27-gauge hypodermic needle into a lateral tail vein under direct visualization.

Stored images were processed postacquisition to compensate for baseline intrinsic contrast and displayed in either dynamic or cumulative (MIP) format for qualitative and quantitative region-of-interest analysis. Time-intensity curves for each region-of-interest were generated from the dynamic images using the intrinsic Vevo 770 software (v.2.23).

Immunohistochemical Staining

Fixed tumors were paraffin-embedded and sectioned for immunostaining. Immunostaining was done with the use of optimized standard protocols on a Ventana Discovery XT instrument (Ventana Medical Systems) using antibodies against phosphorylated ERK1/2 (Ventana Medical Systems), phosphorylated JNK (1:100; Cell Signaling), and phosphorylated p38 MAPK (1:50; Cell Signaling) as well as mouse antibodies for the endothelial cell marker CD31 (1:50; Neomarkers). Horseradish peroxidase-conjugated UltraMap anti-mouse IgG (Ventana Medical Systems) or UltraMap anti-rabbit IgG (Ventana Medical Systems) was used with diaminobenzidine to stain the slides. Tumor cell proliferation was assayed by probing with rabbit antibody against human Ki-67 (1:50; Abcam) and then staining with a biotinylated anti-rabbit secondary antibody

(1:100; Jackson Labs) and horseradish peroxidase-conjugated UltraMap anti-rabbit IgG (Ventana Medical Systems). Apoptosis and necrosis were assayed using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining kit (Promega) according to the manufacturer's instructions.

All of the slides were scanned at $\times 20$ by ScanScope (Aperio Technologies) using the software ScanScope Console v.7.00.08.1020/Controller v.7.00. Images of the whole slides or representative fields were exported by using software Aperio ImageScope v.7.1.33.1025.

Mean Vessel Density

To calculate mean vessel density, images were acquired using a Nikon Eclipse 80i with a spectral Nuance camera (CRI) and analyzed using software developed by our laboratory that provides a simple, cross-platform graphical user interface for image analysis and the ability to implement analysis routines as plug-ins. Immunohistochemical staining intensity was quantified as $255 - i_g$, where i_g is the average intensity of the green channel in the image (expressed as a value ranging from 0 to 255). Stained vessels were counted in the images by automatically thresholding (15) the images produced by the Nuance multispectral imaging system and then applying the connected component (16) to identify contiguous objects (vessels) in the image. Objects less than 100 square pixels in area were considered background noise and were eliminated from the analysis.

Statistics

Data are presented as mean \pm SD. Data were analyzed for significance by a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test or analyzed by a two-tailed Student's *t* test as indicated. $P < 0.05$ was considered significant.

Results

ERK and p38 MAPK Activity Is Reduced following LeTx Treatment of Fibrosarcoma-Derived Cell Lines

Although lethal factor efficiently cleaves and inactivates recombinant MKKs *in vitro*, its effects in cells may be restricted by additional factors including toxin receptor expression. To confirm that LeTx can enter the fibrosarcoma-derived cells used in this study, we treated cells with protective antigen plus E687C or active lethal factor (LeTx) and assayed MKK proteolysis by probing with antibodies against the NH₂ terminus of MEK1. We observed that treatment of cells with protective antigen plus lethal factor, but not E687C, resulted in the loss of NH₂-terminal epitopes of MEK1 [Fig. 1A (HT-1080 line); Supplementary Fig. S1⁷ (NCI and Shac lines)]. This observation is consistent with the explanation that protective antigen is able to bind and facilitate entry of lethal factor into these cells.

MKKs phosphorylate and activate MAPK (that is, ERK, p38 MAPK, and JNK), so measurements of phosphorylated

⁷ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

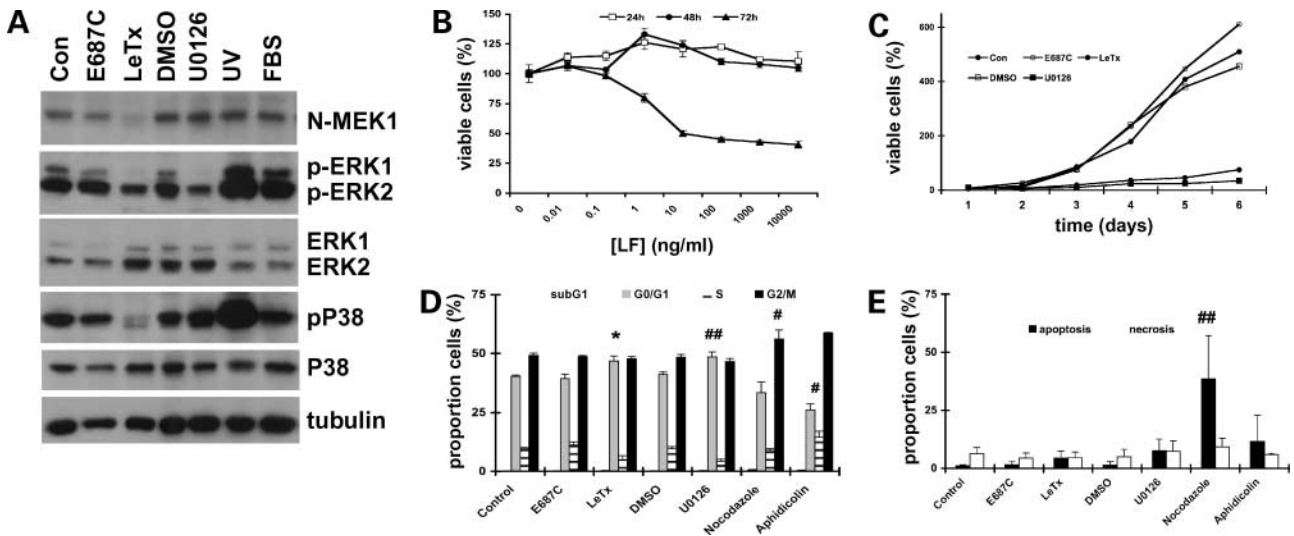


Figure 1. Effects of MKK inhibition on HT-1080 cells *in vitro*. **A**, MKK signaling pathways were inhibited by LeTx treatment. Immunoblotting of lysates from human fibrosarcoma cell line HT-1080 shows loss of phosphorylated ERK and p38 MAPK following treatment with LeTx but no loss in control cells. Cells were treated with medium alone (*con*), 1 $\mu\text{g}/\text{mL}$ protective antigen plus 100 ng/mL E687C, protective antigen plus lethal factor (1 $\mu\text{g}/\text{mL}$ protective antigen plus 100 ng/mL lethal factor; LeTx), DMSO (0.1%), or U0126 (10 $\mu\text{mol}/\text{L}$ from a 10 mmol/L stock in DMSO). The positive (fetal bovine serum) control for phosphorylated ERK was HT-1080 cells activated by 20% fetal bovine serum for 5 min after overnight serum starvation; for phosphorylated p38, cells treated by UV light for 1 min. **B**, inhibition of human fibrosarcoma cell line HT-1080 proliferation by LeTx for 24, 48, or 72 h. Cells were cultured in 96-well plate format and treated with 1 $\mu\text{g}/\text{mL}$ protective antigen plus 0 to 10,000 ng/mL lethal factor for 24, 48, or 72 h. Proliferation was assayed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. At least three experiments were done, each in triplicate assay. Results are expressed as a percentage of the proliferation of control cells treated with protective antigen alone. Bars, SD. **C**, effects of sustained LeTx treatment. The proportion of viable cells in treated versus control cell cultures was assessed following sustained (1-6 d) MKK inhibition by 10 ng/mL LeTx or 10 $\mu\text{mol}/\text{L}$ U0126. Proliferation was assayed as described above. The experiment was repeated in triplicate three times. Representative result. **D**, effects of LeTx on cell cycle distribution of human fibrosarcoma cell lines HT-1080. Response of the human fibrosarcoma cell line HT-1080 to LeTx was analyzed by flow cytometric analysis following PI staining. The cells were starved overnight and then treated with medium alone, 1 $\mu\text{g}/\text{mL}$ protective antigen plus inactive 100 ng/mL lethal factor (E687C), protective antigen plus lethal factor (1 $\mu\text{g}/\text{mL}$ protective antigen plus 100 ng/mL lethal factor), DMSO (0.1%), U0126 (10 $\mu\text{mol}/\text{L}$), nocodazole (0.05 $\mu\text{mol}/\text{L}$), or aphidicolin (0.5 $\mu\text{mol}/\text{L}$) for 24 h. **E**, effects of LeTx on apoptosis of human fibrosarcoma cell line HT-1080. The ability of LeTx to induce apoptosis in HT-1080 cells was analyzed by flow cytometric analysis following Annexin V/PI staining. The cells were treated as described in **C** for 72 h. Bars, SD (**C** and **D**). *, $P < 0.05$; **, $P < 0.01$, compared with E687C-treated cells; #, $P < 0.05$; ##, $P < 0.01$, compared with DMSO-treated cells.

MAPK are an indirect indication of MKK activity. Therefore, to confirm that LeTx can inhibit MKK signaling in these cell lines, we probed lysates of LeTx-treated fibrosarcoma cells with antibodies against phosphorylated MAPK. U0126, a small-molecule inhibitor of MEK1 and MEK2, was used as a positive control for ERK inhibition. We observed that whereas the levels of total ERK and p38 were similar under all conditions tested, levels of phosphorylated ERK and p38 MAPK decreased in response to LeTx but not in response to E687C (Fig. 1A; Supplementary Fig. S1). By contrast, U0126 inhibited only ERK phosphorylation. Although we were able to detect phosphorylated JNK in our UV-treated positive control cells, we were not able to detect it in any of these three fibrosarcoma-derived cell lines under routine cell culture conditions (data not shown). Collectively, these results indicate that LeTx can inhibit MKK signaling in these fibrosarcoma-derived cell lines.

MKK Signaling Is Required for Cell Proliferation *In vitro*

MKK signaling pathways, especially the ERK pathway, play an important role in the proliferation of carcinoma cells *in vitro* (17). Indeed, for some carcinomas such as

melanoma, cell survival is dependent on MKK signaling (18, 19). To determine whether the same holds for fibrosarcoma-derived cells, we cultured cells in 96-well plates in the presence or absence of LeTx for 24, 48, or 72 h, after which we measured the relative number of viable cells; for comparison, we tested the effects of U0126. LeTx had only a limited effect on fibrosarcoma cell proliferation over the course of 24 to 48 h (Fig. 1B; Supplementary Fig. S2A-C). However, after 72 h, LeTx treatment inhibited cell proliferation by as much as 50%. The amount of LeTx required for a half-maximal effect on growth (GI_{50}) was 1.3 ± 0.2 ng/mL. By comparison, the GI_{50} for melanoma cells is 0.03 ng/mL (20). Similarly, U0126 inhibited cell proliferation after 48 to 72 h of treatment (Supplementary Fig. S2D-F), with a GI_{50} of ~ 10 $\mu\text{mol}/\text{L}$. Sustained treatment of HT-1080 cells with either 10 ng/mL LeTx or 10 $\mu\text{mol}/\text{L}$ U0126 caused an $\sim 90\%$ reduction in the proportion of viable cells compared with control cultures (Fig. 1C). These results indicate that sustained inhibition of MKK signaling, particularly MEK1 and MEK2, leads to a decrease in the viable number of fibrosarcoma cells.

Previously, we showed that treatment of melanoma cells with LeTx initially causes G_1 arrest that is followed at

72 h by the induction of apoptosis (19). Accordingly, we used fluorescence-activated cell sorting analysis to determine whether the observed decrease in the number of viable HT-1080 fibrosarcoma cells was caused by decreased proliferation, increased cell death, or a combination of both. As positive controls, we included nocodazole, a microtubule-destabilizing agent that arrests cell cycle progression at G₂-M phase (21), and aphidicolin, an inhibitor of DNA replication that arrests cell cycle progression in S phase (22). Cells were first serum-starved overnight to semisynchronize cell cycle progression and then were restored to culture medium containing serum plus inhibitor. Twenty-four hours later, the DNA of cells was stained with PI and their cell cycle profile was measured by fluorescence-activated cell sorting analysis. For all three cell lines tested, cells treated with inactive LeTx or DMSO showed a cell cycle profile that was indistinguishable from cells treated with medium alone (Fig. 1D; Supplementary Fig. S3A and B). As expected, treatment with nocodazole or aphidicolin caused varying degrees of delay in cell cycle progression at G₂-M or S phase, respectively, in all three cell lines. In contrast, both LeTx and U0126 caused a modest (5-10%) but statistically significant increase in the proportion of cells in G₁ phase without a detectable increase in sub-G₁ content.

As an alternative approach to determining whether the observed decrease in the number of viable HT-1080 fibrosarcoma cells was caused by increased cell death, we measured rates of apoptosis by PI/Annexin V staining of cells treated for 72 h with LeTx or U0126. Annexin V is a Ca²⁺-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine and is useful for identifying apoptotic cells. PI was used to distinguish viable from necrotic cells; viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Whereas nocodazole and aphidicolin caused increased Annexin V staining of HT-1080 cells after 72 h of treatment, LeTx and U0126 had no measurable effect (Fig. 1E; Supplementary Fig. S3C and D). Only modest changes in PI staining were noted for LeTx or U0126. Collectively, these results indicate that inhibition of MKK signaling by treatment with LeTx or U0126 largely decreases the number of viable cells by decreasing the rates of proliferation without measurably increasing rates of cell death.

MKK Signaling Is Required for Growth of Fibrosarcoma-Derived Xenografts

To test the requirement for MKK signaling in fibrosarcoma growth *in vivo*, we did xenograft studies with athymic nude mice. HT-1080, NCI, or Shac cells were injected s.c. into the dorsal flank of 24 mice. Fibrosarcomas were permitted to grow until they attained an average volume of ~100 mm³, at which point the mice were randomly divided into four groups and treated every second day with i.v. injections of 0.5 to 2 standard doses of LeTx (1 standard dose = 10 µg protective antigen and 2 µg lethal factor in a volume of 50 µL) or with two standard doses protective antigen plus E687C as a negative control. For all

three cell lines, four to six systemically administered doses of LeTx reduced fibrosarcoma growth by as much as 50% to 75% [Fig. 2A (HT-1080); Supplementary Fig. S4A and B (NCI and Shac)]. Maximal effects were observed at 1 standard dose.

To verify target inhibition and to provide insight into the manner in which tumor growth was inhibited, we did immunostaining of formalin-fixed tumor samples with antibodies raised against active MAPK as well as with antibodies against markers of proliferation and of cell death. Control tumors that were treated with protective antigen plus E687C contained viable, proliferating cells throughout the tumor (Fig. 2B). Regions of active MAPK signaling were evident (Fig. 2C). By contrast, comparison of staining in serial sections of LeTx-treated tumors showed extensive (>70%) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive regions in the stroma, whereas proliferating cells were restricted to the cortex. Similarly, although regions of MAPK activity were evident in LeTx-treated tumors, they were restricted to the cortex. These results indicate that MKK inhibition by LeTx is accompanied by decreased tumor cell proliferation and increased tumor cell death.

MKK Signaling Is Required for Vascularization of Fibrosarcoma-Derived Xenografts

The central pattern of tumor death elicited by LeTx is very similar to that seen with antiangiogenic agents (23–25). Indeed, macroscopic/visual comparison of LeTx-treated xenograft tumors suggested that their vascular content was decreased in comparison with that of control E687C because they were colored pale tan versus deep purple (Supplementary Fig. S5). To test whether LeTx caused a reduction in vascular content, we did immunostaining of formalin-fixed tumor samples with antibodies raised against the endothelial marker CD31. These slides were then used to calculate mean vessel densities of both toxin-treated and control fibrosarcomas. Compared with the tumors treated with E687C, the mean vessel densities of LeTx-treated tumors were decreased in a dose-dependent manner by as much as 70% ($P < 0.0001$; Fig. 3A and B). These results indicate that MKK signaling is essential for vascularization of fibrosarcoma xenografts.

MKK Signaling Is Required to Maintain Tumor Blood Flow

As an alternative approach to test the requirement for MKK signaling in fibrosarcoma vascularization *in vivo*, we monitored the tumor perfusion in xenografts using ultrasound imaging in conjunction with injecting contrast ultrasound microbubbles. HT-1080 cells were injected in athymic nude mice to form xenograft tumors. When tumors attained a volume of ~100 mm³, we administered by tail vein injection either 1 standard dose of either LeTx or protective antigen plus inactive lethal factor as a negative control. Before treatment, ultrasound imaging revealed a diffuse pattern of blood flow throughout the stroma and cortex of the tumor xenograft (Fig. 4A). By contrast, blood flow-through tumors as assessed by microbubble content was markedly diminished within 24 h of LeTx treatment,

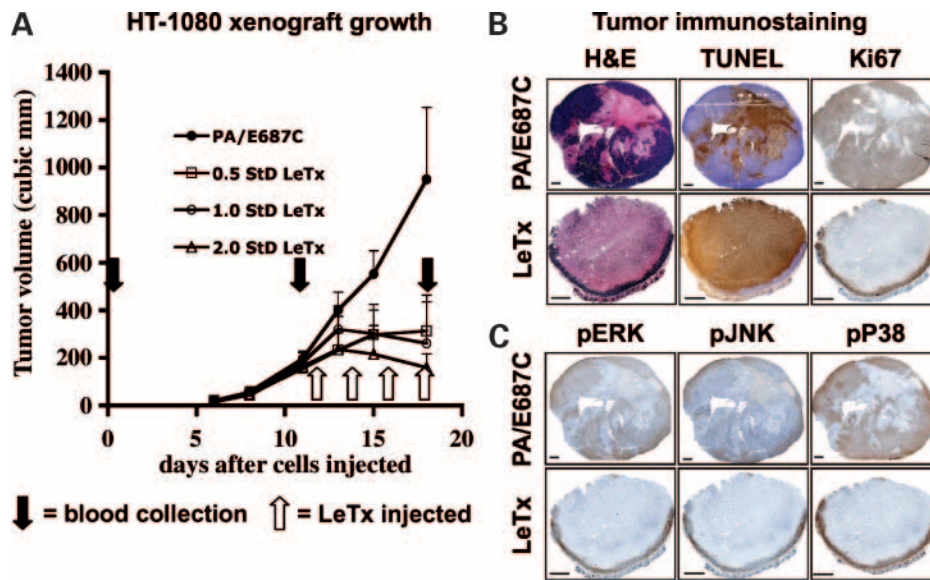


Figure 2. Effects of MKK inhibition on tumor growth. **A**, LeTx inhibited the growth of HT-1080 xenograft tumors. Tumor volume is plotted as a function of time (days after cells initially injected). When HT-1080 fibrosarcoma xenograft tumors attained a size of 100 mm³, they were treated with 2 standard doses of protective antigen plus E687C (filled diamonds) or 0.5, 1, or 2 standard doses of LeTx (empty triangles, filled triangles, or X marks, respectively) by i.v. injection ($n = 6$). Doses were administered at 2-day intervals for 1 to 2 weeks. Open arrows, time of treatment; solid arrows, days on which blood samples were collected. Bars, SD. **B**, LeTx inhibition of MAPK phosphorylation is accompanied by decreased tumor cell proliferation and cell death. Treated tumor tissues (1 standard dose LeTx or protective antigen/E687C controls) were excised 24 h after the final treatment, fixed in formalin, sectioned, and immunostained with antibodies to the apoptosis/necrosis marker terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, the proliferation marker Ki67, or the active, phosphorylated forms of ERK, JNK, and p38 MAPK, or else stained with H&E. Note: LeTx-treated tumors were imaged at higher magnification ($\times 10$ versus $\times 4$). Bar, 1 mm (for all images).

whereas flow-through tumors treated with protective antigen and E687C or flow-through normal (kidney) tissues treated with LeTx was unaffected (Fig. 4B and C; Supplementary Videos). These results indicate that inhibition of MKK signaling by LeTx causes a rapid and dramatic decrease in tumor perfusion.

To determine how LeTx causes this decrease in tumor perfusion, we did immunohistochemistry on paraffin sections from tumor and kidney tissue that was fixed in formalin 24 h after treatment with 1 standard dose LeTx or protective antigen plus inactive lethal factor immediately

following ultrasound observations. Using H&E staining, we observed that whereas tumors treated with protective antigen plus inactive lethal factor appeared unremarkable (Fig. 5A and B), tumors treated with LeTx contained large regions of hemorrhage (Fig. 5C and D). No hemorrhage was observed in kidney tissues treated with LeTx (Fig. 5F). To determine whether 24-h LeTx treatment decreased tumor vascularization, we immunostained tumors with antibodies against CD31. Quantification of this staining showed LeTx induced only a small, statistically insignificant ($P = 0.3$) decrease in mean vascular density when

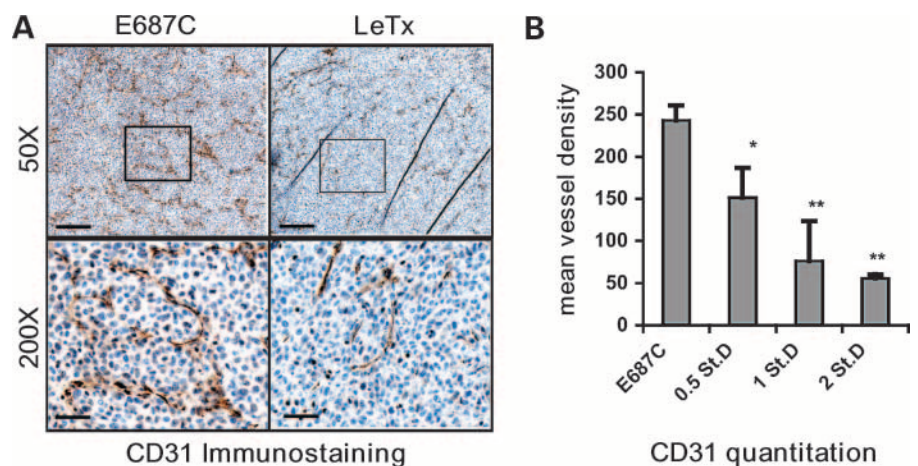


Figure 3. MKK signaling pathway was required for the tumor angiogenesis *in vivo*. **A**, tissue sections of HT-1080 xenograft tumors treated with E687C or LeTx were immunostained with antibodies to the endothelial marker CD31 at low (top; $\times 50$; bar, 200 μ m) and high (bottom; $\times 200$; bar, 50 μ m) magnification. **B**, mean \pm SD vessel density was calculated using five fields from each of three separate tumors. *, $P < 0.05$; **, $P < 0.01$, compared with E687C-treated samples.

compared with tumors that were treated with protective antigen plus inactive lethal factor. These results indicate that the rapid and dramatic decrease in tumor perfusion caused by LeTx is primarily caused by increased extravasation.

MKK Signaling Is Required for Cytokine Release *In vitro*

Tumor vascularization is driven by the release of tumor-derived angioproliferative growth factors that induce vasculogenesis and/or the remodeling of existing blood vessels to support the growing tumor. To test whether MKK signaling was required for the release of angioproliferative growth factors from fibrosarcoma-derived cell lines, we next examined the effects of LeTx on cytokine release from these cells *in vitro* using ELISA. For comparison, we also examined the effect of U0126. Whereas LeTx caused a substantial (>75%) inhibition of VEGF release within 24 h from each fibrosarcoma-derived cell line, U0126 inhibited the release of VEGF from the HT-1080 and Shac cell lines but not from the NCI cells [Fig. 6A (HT-1080); Supplementary Fig. S6 (NCI and Shac)]. These results indicate that MKK signaling is necessary for release of VEGF from fibrosarcoma-derived cell lines most likely

through MEK1 and MEK2 (but other MKK signaling pathways may also play a role in this process in a cell type-specific fashion).

To determine whether the requirement for MKK signaling was specific for VEGF release or was a general requirement for cytokine release, we assayed the effects of LeTx on the release of a panel of human cytokines from HT-1080 cells (Table 1; Supplementary Material). Our analysis revealed that both LeTx and U0126 decreased the release of a number of angioproliferative factors, including bFGF, IL-8, and VEGF, the same cytokines that have been noted as strong correlates of disease-free and overall survival in other tumors such as melanoma (26), but this effect was far from global as the release of several cytokines including α -fetoprotein and tumor necrosis factor- α was unaffected. These observations indicate that MKK signaling promotes the release of multiple angioproliferative cytokines.

To confirm that a similar relationship among MKK signaling, angioproliferative cytokine release, and tumor growth and vascularization exists *in vivo*, we used anti-human antibodies and measured levels of cytokines (focusing on bFGF, IL-8, and VEGF) in the serum of tumor-free mice and mice bearing HT-1080 fibrosarcoma

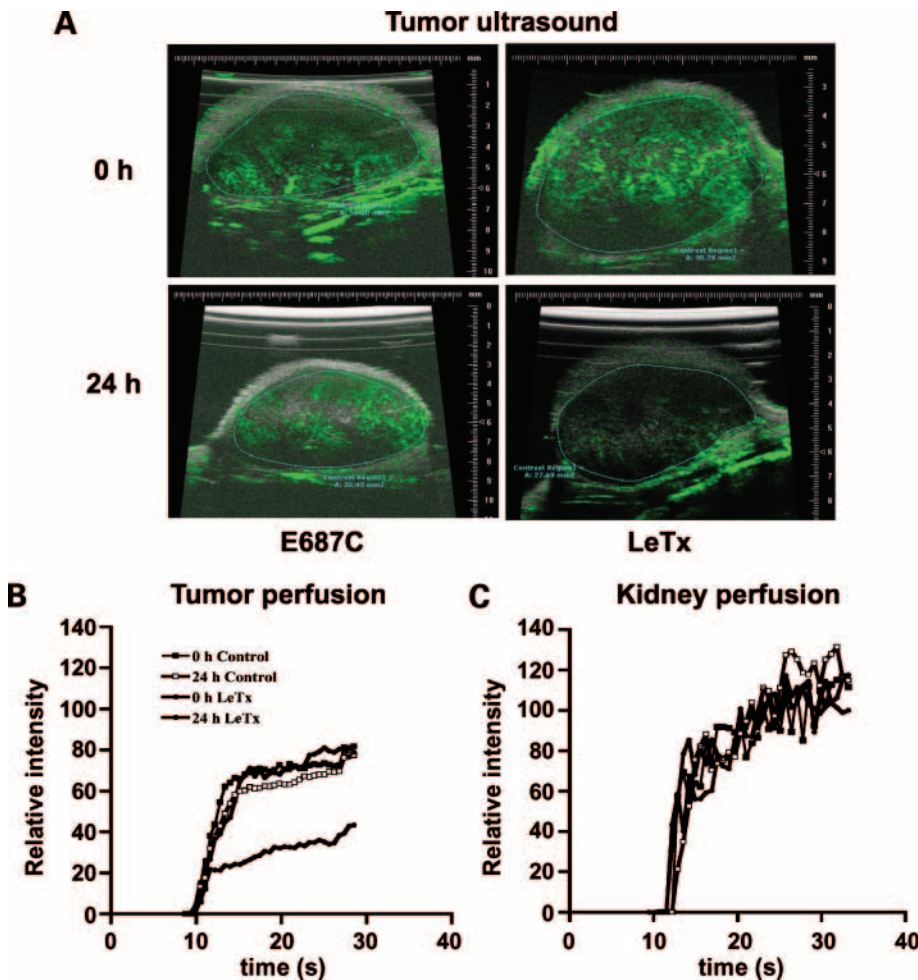


Figure 4. Ultrasound analysis of the effects of acute MKK inhibition on tumor blood flow. HT-1080 fibrosarcoma xenograft tumors (~100 mm³ in diameter) were treated with 1 standard dose of either E687C or LeTx by i.v. injection ($n = 3$). Tumor perfusion was evaluated by ultrasound imaging enhanced with contrast microbubbles either immediately before treatment or 24 h after treatment. **A**, contrast signals (green spots) are proportional to the number of microbubbles within the region of interest, which in turn reflect the included volume of flowing blood. Relative changes in contrast signal intensity over time in tumor tissue (**B**) or kidney (**C**) are shown by curves generated from the dynamic imaging sequence. Filled and empty triangles, values from tumors just before or 24 h after E687C treatment, respectively; filled and empty circles, values from tumors just before or 24 h after LeTx treatment, respectively. Each curve represents the average contrast signals of three mice from each group.

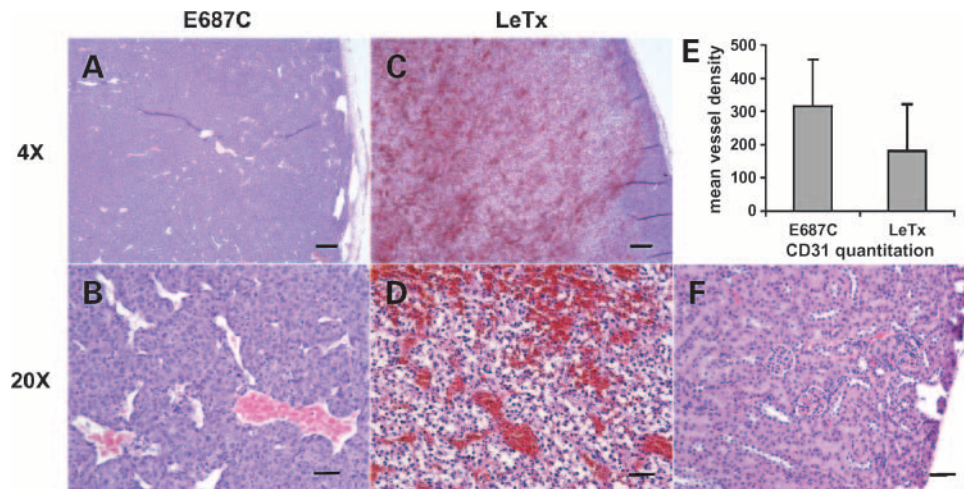


Figure 5. Effect of acute MKK inhibition on xenograft morphology and vascularity. Mice bearing HT-1080 xenograft tumors were injected i.v. with 1 standard dose protective antigen plus inactive lethal factor (**A** and **B**) or LeTx (**C** and **D**). Twenty-four hours later, ultrasound measurements were done. Immediately, tumors or kidney tissue (**F**) were removed from the mice. These tissues were formalin fixed, paraffin embedded, sectioned, and stained using H&E staining. Images were obtained at $\times 4$ (**A** and **C**) or $\times 20$ (**B**, **D**, and **F**). Bar, $200\ \mu\text{m}$ (**A** and **C**) or $50\ \mu\text{m}$ (**B**, **D**, and **F**). **E**, sections of tumors treated for 24 h with 1 standard dose protective antigen plus inactive lethal factor or LeTx were stained with anti-CD31 (not shown). CD31 staining intensity was quantified as described in Fig. 3. Mean \pm SD vessel density was calculated using CD31 quantitation in five fields from each of three separate tumors.

xenografts before and after treatment with protective antigen plus inactive lethal factor or LeTx. None of these cytokines were detected in mice before tumor cell injection (data not shown). This suggests either that mice cytokines do not cross-react with anti-human antibodies or that these cytokines are not normally expressed in mice. Whereas levels of bFGF were comparable in mice treated with inactive or active LeTx (40.9 ± 33.1 versus 31.6 ± 35.8 pg/mL, respectively), levels of IL-8 and VEGF were markedly reduced in mice treated with active LeTx (IL-8, 1323 ± 199 versus 317 ± 101 pg/mL; VEGF, 406 ± 77 versus 82 ± 13 pg/mL). LeTx also inhibited the release of other cytokines whose expression has been linked to aspects of tumor growth and vascularization, including

granulocyte-macrophage colony-stimulating factor (189 ± 23 versus 46 ± 16 pg/mL) and MCP-1 (560 ± 135 versus 206 ± 103 pg/mL).

To control for the effects of LeTx on host cytokine expression, we examined the same serum samples with mouse-specific antibodies. The levels of murine VEGF were comparable in mice lacking tumors (392 ± 210 pg/mL) and those with tumors that had been treated with inactive (429 ± 112 pg/mL) or active LeTx (341 ± 118 pg/mL). Similarly, circulating levels of bFGF were comparable in mice lacking tumors (1.7 ± 0.3 pg/mL) and those with tumors that had been treated with inactive (1.8 ± 0.2 pg/mL) or active LeTx (2.0 ± 0.5 pg/mL). We did not detect KC/GRO α (murine IL-8; threshold for detection = 0.2 ng/mL) in

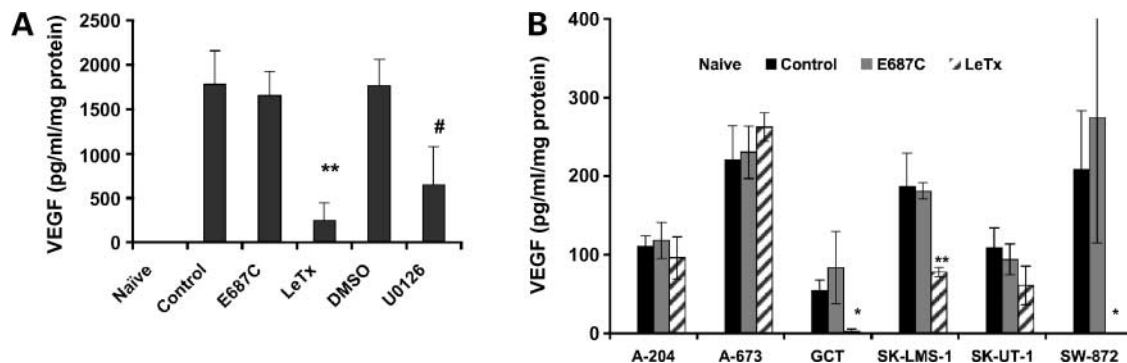


Figure 6. Effect of MKK inhibition of VEGF release from fibrosarcoma- and sarcoma-derived cell lines. **A**, HT-1080 cells were treated as described and VEGF levels in conditioned medium were measured by ELISA. Results are normalized to the amount of protein present in each culture dish. Columns, average of at least three independent experiments; bars, SD. "Naive medium" group shows VEGF in culture medium (containing 10% fetal bovine serum) that has not been exposed to cells. **B**, effect of LeTx on the VEGF release from human sarcoma-derived cell lines. Sarcoma-derived cell lines were treated with medium alone (control), $1\ \mu\text{g/mL}$ protective antigen plus inactive $100\ \text{ng/mL}$ lethal factor (E687C), or protective antigen plus lethal factor ($1\ \mu\text{g/mL}$ protective antigen plus $100\ \text{ng/mL}$ lethal factor, LeTx) and VEGF content of the medium was measured by ELISA at 24 h as described. Results are an average of three experiments; bars, SD (for all experiments). *, $P < 0.05$; **, $P < 0.01$, compared with E687C; #, $P < 0.05$, compared with DMSO.

Table 1. Screening of *in vitro* cytokine release

Analyte	Untreated	Inactive LeTx	Active LeTx	DMSO	U0126
bFGF (pg/mL)	120 ± 9	128 ± 2	29 ± 5	127 ± 19	66 ± 20
IL-8 (ng/mL)	4.1 ± 1.8	3.4 ± 1.0	0.2 ± 0.0	4.0 ± 1.2	0.3 ± 0.1
IL-6 (pg/mL)	550 ± 18	720 ± 295	123 ± 102	535 ± 182	222 ± 167
IL-7 (pg/mL)	8.0 ± 1.6	4.5 ± 2.4	< ld	7.6 ± 2.6	< ld
MMP-2 (ng/mL)	56.1 ± 5.4	57.9 ± 10.2	< ld	58.0 ± 7.1	< ld
VEGF (ng/mL)	6.8 ± 0.3	7.0 ± 1.3	0.9 ± 0.3	7.5 ± 0.9	2.4 ± 1.1
α-Fetoprotein (ng/mL)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
MMP-3 (ng/mL)	0.67 ± 0.19	0.63 ± 0.21	0.91 ± 0.07	0.64 ± 0.16	1.14 ± 0.21
Tumor necrosis factor-α (pg/mL)	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.2	1.4 ± 0.5

NOTE: Conditioned medium was collected from HT-1080 cells cultured for 24 h in the presence of inactive or active LeTx. Results are expressed as an average ± SD of at least three independent experiments. < l d, beneath the limits of detection.

serum samples. Collectively, these results indicate that MKK signaling is necessary for release of a select group of proangiogenic cytokines from fibrosarcoma-derived cell lines *in vitro* and *in vivo*.

MKK Signaling Is Required for the Release of Proangiogenic Factors from Other Sarcoma-Derived Cell Lines

Finally, we asked whether MKK signaling was required for the release of angioproliferative cytokines from other types of sarcomas. The GCT cell line (human fibrous histiocytoma), the SK-LMS and SK-UT-1 cell lines (human leiomyosarcoma), the SW-872 cell line (human liposarcoma), and the A-673 and A-204 cell lines (human rhabdomyosarcoma) were grown in culture for 24 h in the presence or absence of LeTx. Then, we examined the conditioned medium from these cell cultures for VEGF levels by ELISA and made lysates of the cells to confirm that LeTx was able to enter these cells and proteolytically modify MKKs (data not shown). The results of this analysis indicate that LeTx significantly reduced the release of VEGF from the GCT, SW-872, and SK-LMS cell lines but not from the SK-UT-1, A-673 or A-204 lines (Fig. 6B). These results show that MKK signaling is required for the release of angioproliferative cytokines from not just fibrosarcoma-derived cell lines but also some cell lines derived from malignant fibrous histiocytomas, leiomyosarcomas, and liposarcomas.

Discussion

VEGF-A (hereafter called VEGF) plays a crucial role in regulating tumor angiogenesis (27). *In vitro*, VEGF is a potent mitogen of endothelial cells (28, 29). *In vivo*, intradermal injection of VEGF causes increased vascular permeability (30). Inhibition of VEGF release from tumors leads to decreased tumor vascularization and growth (31) and embryonic stem cells that are homozygous null for VEGF are unable to form tumors in nude mice (32).

Several studies indicate that MKK signaling pathways act to coordinate not only the release of VEGF but also the response to VEGF. MKK activity regulates VEGF expres-

sion at the transcriptional and post-transcriptional levels (33). Expression of constitutively activated MEK1 in fibroblasts elevates the expression of VEGF mRNA through binding of the transcription factors Sp1 and AP-2 to its promoter region (34). Also, VEGF mRNA half-life is increased in cells that overexpress p38 MAPK and JNK (33). MKK signaling pathways are also activated in response to VEGF; the treatment of endothelial cells with VEGF has been observed to cause activation of both ERK1 and ERK2 (35) as well as p38 MAPK (36). Moreover, this increase in MKK activity is required for endothelial response to VEGF. Whereas MEK1/2 inhibitors prevent VEGF-induced cell proliferation (36, 37), p38 MAPK inhibitors reportedly prevent VEGF-induced cytoskeletal reorganization and cell migration (36). The JNK pathway has also been linked to *in vitro* endothelial cell motility (38, 39) and proliferation (38).

Despite evidence of a strong link between MKK signaling, endothelial cell function, and the release of angioproliferative factors *in vitro*, the role MKKs play in promoting tumor vascularization *in vivo* is less clear. Insight into their *in vivo* functions may be inferred from the effects of MKK inhibitors on tumor vascularization. For example, we have shown previously that anthrax lethal factor substantially inhibits vascularization in mouse xenograft studies (11, 40). Also, expression of an inactive Raf-1 mutant in endothelial cells blocks the growth and vascularization of melanomas in mice (41). BAY 43-9006 (sorafenib), a compound that inhibits B-Raf and C-Raf (MKKs that activate MEK1 and MEK2), also reduces tumor vascularization *in vivo* (42). However, whether this is due directly to inhibition of MKK signaling is unclear because (a) an antiangiogenic effect has not been reported for other MEK1 and MEK2 inhibitors such as CI1040 (PD184352) and (b) BAY 43-9006 can also inhibit the activity of the VEGF receptor, VEGFR2 (42). Finally, Mavria et al. (43) have shown that the expression of dominant-negative MEK1 in the vasculature of colorectal adenocarcinoma xenografts suppresses angiogenesis and tumor growth.

In this study, we have presented data that indicates MKKs also play a significant role in the growth of

fibrosarcomas. Although *in vitro* MKK inhibition caused only a modest decrease in cell proliferation up to 48-h treatment, it had a significant effect at later stages. In addition, LeTx caused a significant reduction of the release of key angioproliferative cytokines such as VEGF, IL-8, and bFGF. Available evidence indicates that some of the same angioproliferative cytokines are essential for the growth, vascularization, and metastasis of sarcoma. Analyses of serum samples from sarcoma patients shows a stage-dependent increase of angioproliferative cytokines including bFGF, IL-6, VEGF, macrophage colony-stimulating factor, and tumor necrosis factor receptor I (44, 45), with decreased levels of IL-6, IL-8, and tumor necrosis factor receptor II as prognostic correlates of disease-free and overall survival (44). Based on these results, we propose that inhibition of MKK signaling causes a decrease in cytokine release that normally promote tumor cell proliferation in an autocrine fashion. An alternative hypothesis that MKK inhibition leads to the accumulation of intrinsic signals that cause tumor cell death seems less likely as fluorescence-activated cell sorting cell cycle analysis shows LeTx-treated cells begin to accumulate at the G₁-S transition (Fig. 1C) after 24-h treatment without change in the incidence of apoptosis or necrosis over 72 h (Fig. 1D).

In either case, we would have predicted that *in vivo* administration of LeTx would lead to reduced tumor growth over the course of several days of treatment. However, we observed that the rate of tumor growth declined within 48 h of the first LeTx injection (Fig. 2A). Moreover, ultrasound measurement of tumor blood flow showed a dramatic loss of perfusion likely due to extravasation within 24-h treatment (Fig. 4). This was unexpected because a previous study has shown that withdrawal of angiogenic stimuli leads to a regression of neovascularization over the course of weeks, not hours (46). Endothelial survival during angiogenesis is strictly dependent on their interaction with the extracellular matrix via integrins (reviewed in ref. 47). Perhaps the simultaneous loss of tumor-derived proangiogenic factors and the inability of endothelial cells to respond to those factors in a MKK-dependent fashion results in decreased extracellular matrix binding that causes a catastrophic loss of vascular activity. Our failure to observe similar changes in normal endothelium indicates that the survival requirements for normal and tumor endothelium are distinct. Taken together, our results indicate that although MKK activity is required for tumor cell proliferation, it also plays an important role in promoting tumor vascular function. Further studies are required to delineate the events leading to loss of vascular function as well as the relative contributions of tumor, stromal, and endothelial cells in this response.

Although our studies indicate that LeTx and U0126, a small-molecule inhibitor of MEK1 and MEK2, have similar effects on cytokine release *in vitro*, it is curious that an antiangiogenic effect for MEK1/2 inhibitors has not been reported *in vivo*. We offer three explanations for this discrepancy. First, the anthrax toxin receptors are up-

regulated in tumor-associated endothelium (48, 49). Second, binding to toxin receptors by protective antigen alone, albeit at levels in excess of those used in this study, has an antiangiogenic effect (50). Third, LeTx causes broad MKK inhibition. The latter point is significant because mouse knockout studies have shown that not only MEK1 and ERK2 but also other members of the MAPK signaling pathways including MEKK3 and p38 α MAPK are essential for developmental vascularization (51). These observations indicate that use of LeTx may have an advantage versus small-molecule MEK1/2 inhibitors that are now entering clinical trials.

The results of this and our earlier studies imply that MKK inhibitors such as LeTx may be effective therapeutic agents for the treatment of not only fibrosarcomas but also a broad spectrum of sarcomas and carcinomas, including malignant fibrous histiocytomas, leiomyosarcomas, and liposarcomas; melanoma (19, 20); and Kaposi's sarcoma (40). Based on this, we propose that MKK inhibition by LeTx is a broadly effective strategy for targeting tumor vascularization and by extension other neovascular diseases such as various retinopathies. However, LeTx differs from other antiangiogenic agents in its mechanism of inhibition (27). Not only does MKK inhibition dramatically reduce the release of a broad range of angiogenic regulators, but published data also indicate it prevents response to those regulators. This novel, "two-pronged" strategy may, alone or in combination with conventional antiangiogenic agents, prove to be an effective approach for blocking angiogenesis.

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