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

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Abstract

Purpose: In this study, we tested the hypothesis that inhibition of mitogen-activated protein kinase kinases (MKK) inhibits tumor growth by acting on angiogenic signaling and by extension may form the basis of an effective strategy for treatment of Kaposi's sarcoma.

Experimental Design: Murine endothelial cells expressing the human herpes virus 8 G protein–coupled receptor (vGPCR-SVEC) were treated with anthrax lethal toxin (LeTx). LeTx is a binary toxin ordinarily secreted by *Bacillus anthracis* and is composed of two proteins: protective antigen (the binding moiety) and lethal factor (the active moiety). Lethal factor is a protease that cleaves and inactivates MKKs.

Results: *In vitro*, treatment of vGPCR-SVEC with LeTx inhibited MKK signaling, moderately inhibited cell proliferation, and blocked the ability of these cells to form colonies in soft agar. Treatment with LeTx also blocked the ability of these cells to release several angioproliferative cytokines, notably vascular endothelial growth factor (VEGF). In contrast, inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 with U0126 caused a substantial inhibition of proliferation but only modestly inhibited VEGF release. In xenograft models, i.v. injection of LeTx caused reduced tumor growth characterized immunohistochemically by inhibition of MKK signaling, decreased rates of proliferation, and reduced levels of VEGF and VEGF receptor 2, with a corresponding decrease in vascular density.

Conclusions: These data support a role for MKK signaling in tumor growth and vascularization and are consistent with the hypothesis that inhibition of MKK signaling by LeTx or a similar agent may be an effective strategy for the treatment of Kaposi's sarcoma as well as other vascular tumors.

Anthrax lethal toxin (LeTx) is composed of two proteins, protective antigen (PA) and lethal factor (LF), which are part of the exotoxin secreted by the Gram-positive bacterium *Bacillus anthracis* (1). PA is nontoxic and facilitates LF entry into cells via the endosomal pathway. LF is a Zn^{2+}-metalloproteinase that cleaves and inactivates mitogen-activated protein kinase (MAPK) kinases [MKK or MAPK/extracellular signal-regulated kinase (ERK; MEK)], including MKK1, MKK2, MKK3, MKK4, MKK6, and MKK7 but not MEK5 (2, 3). Because activation of MKK signaling pathways has been shown in tumorigenesis, we earlier tested the ability of LF to inhibit the tumorigenic phenotype of V12 H-Ras–transformed NIH3T3 cells *in vitro* and *in vivo* (4). We noted that LeTx caused morphologic and phenotypic reversion of transformed cells *in vitro* and caused regression of tumor xenografts *in vivo*. Similarly, LeTx has been shown to inhibit the growth of melanoma (5–7). Significantly, LeTx caused a marked reduction of the tumor vascular content of V12 H-Ras–transformed NIH3T3 xenografts (4). The antiangiogenic properties of LeTx were unexpected but may be explained by the subsequent observations that anthrax toxin receptors are up-regulated on tumor endothelial cells (8) and/or by the essential role of MKK signaling in response to vascular endothelial growth factor (VEGF; ref. 9).

We therefore hypothesized that LeTx may form the basis of a novel therapeutic for tumors, particularly those with a high vascular content. Among the latter is Kaposi's sarcoma. Kaposi's sarcoma is a multifocal vascular tumor that is characterized histologically by proliferating spindle cells, angiogenesis, erythrocyte-replete vascular slits, edema, and a variable inflammatory cell infiltrate (10–13). The spindle cells are

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central to the pathogenesis of this tumor and are thought to promote its growth by releasing angioproliferative cytokines that drive the recruitment and proliferation of endothelial cells to the tumor (14–17). The etiologic agent of Kaposi's sarcoma is human herpes virus 8 (HHV8 or KSHV), and expression of the *viral G protein–coupled receptor* (*vGPCR*), a lytic gene from HHV8 (10), is sufficient to induce Kaposi's sarcoma–like tumors in mice (12). Among its functions, vGPCR has been shown to activate MKK signaling pathways (18) to induce VEGF release from NIH3T3 cells (19) and to modulate expression of cytokines in endothelial cells (20). Thus, vGPCR represents a therapeutic target for treatment of Kaposi's sarcoma (21).

In this study, we have used LeTx and vGPCR-expressing mouse endothelial cells [SV40-transformed mouse endothelial cells (SVEC); ref. 12] to test the hypothesis that inhibition of MKK signaling may form the basis of an effective strategy for treatment of Kaposi's sarcoma. We show that LeTx effectively inhibits the tumorigenic properties of vGPCR-SVEC cells and dramatically decreases their ability to release angioproliferative cytokines, such as VEGF. Our results indicate that inhibition of MKK signaling may be an effective strategy for the treatment of Kaposi's sarcoma as well as other tumors with a high vascular content.

Materials and Methods

Cell lines and reagents. Parental SVEC and vGPCR-expressing SVEC (vGPCR-SVEC) were a kind gift from Dr. Silvio Gutkind (National Institute of Dental and Craniofacial Research, Bethesda, MD). Unless noted otherwise, these cells were grown at 37°C in a fully humidified 5% CO_2 atmosphere using DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (plus 0.75 mg/mL geneticin for vGPCR-SVEC only; Invitrogen). PA, inactive LF (E687C), and LF were purified from attenuated cultures of *B. anthracis* as described (22). Stock solutions (10 mmol/L) of the small-molecule MEK1/2 inhibitor U0126 (Calbiochem) were prepared fresh in DMSO.

Immunoblotting. For detection of extracellular signal-regulated kinase (ERK) activation, cells were incubated in serum-free medium alone (control) or in serum-free medium containing DMSO (0.1%), PA (1 μg/mL) + E687C (100 ng/mL), PA + LF (100 ng/mL), or U0126 (10 μmol/L) for 20 h followed by brief (2 min) treatment with 2% fetal bovine serum. Cells were lysed on ice using radioimmunoprecipitation assay buffer. The lysates were centrifuged at 16,110 × *g* in an Eppendorf 5414 benchtop centrifuge for 10 min at 4°C and protein levels of the supernatants were determined using the bicinchoninic acid protein assay (Pierce Biotechnology). Cell lysates were mixed with 2× SDS-loading buffer and boiled 3 min before proteins (5 μg) were separated by 10% Tris-glycine gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked in 5% milk in TBS-Tween 20 followed by overnight incubation at 4°C in rabbit anti-Nterm-MEK1 (1:1,000; Upstate Biotechnology), rabbit anti-Nterm-MEK2 (1:200; Santa Cruz Biotechnology), rabbit anti-p44/42-MAPK (1:1,000; Cell Signaling Technology), mouse anti-α-tubulin (DM1A; 1:1,000; Sigma), or mouse anti-phosphorylated p44/42-MAPK, p38 MAPK, phosphorylated p38 MAPK, c-Jun NH_2-terminal kinase (JNK), phosphorylated JNK, ERK5, or phosphorylated ERK5 (1:1,000; Cell Signaling Technology). Then, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies at 1:2,000 (KPL). Specific proteins were visualized using X-ray films after incubation with enhanced chemiluminescence reagents (Cell Signaling Technology).

Proliferation assays. Cells were cultured in 96-well plates in the presence of PA (1 μg/mL) and various concentrations of LF (0-10,000 ng/mL) for 24, 48, and 72 h. The relative number of viable cells at the end of this period was assayed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Data are presented relative to proliferation of control samples incubated in the presence of medium alone and as an average of three independent experiments (±SD).

Soft agar colony formation. vGPCR-SVEC cells were seeded in six-well plates (5×10^5 per well) with culture medium and 0.5% (w/v) SeaPlaque agar (Cambrex Corp.). After 24 h, the cells were incubated in medium alone or in medium containing U0126 (10 μmol/L), DMSO (0.1%), or PA (1 μg/mL) plus either E687C (100 ng/mL) or LF (100 ng/mL). The medium was changed out every 24 h for 2 weeks. At the end of the 2 weeks, colonies >0.5 mm in three random fields of each treatment plate were counted. The results represent the average of three independent experiments ± SD.

ELISA. Conditioned medium was collected and mixed with conditioned medium buffer [250 mmol/L HEPES, 1% bovine serum albumin, 10 mmol/L EDTA (pH7.4)] supplemented with protease inhibitors (Complete EDTA-free, Roche Diagnostics). Conditioned medium was then filtered (0.22 μm; Millipore) and frozen at -86°C until assayed by ELISA for VEGF (Calbiochem). Additional assays for cytokine secretion were done by Pierce Biotechnology. All results were normalized to the protein content of each dish and represent at least three independent experiments (±SD). For phosphorylated ERK quantification, cells were lysed in radioimmunoprecipitation assay buffer as described and analyzed using a pERK ELISA kit (Assay Designs). Results were normalized to cell number and represent three independent experiments (±SD).

In vivo *tumorigenicity assays*. All experiments were done in compliance with the principles of the Guide for the Care and Use of Animals. In addition, all procedures were approved before use by the Institutional Animal Care and Use Committee of the Van Andel Research Institute. vGPCR-SVEC cells (3×10^6 in a volume of 100 μL) were injected s.c. into the dorsal flank of 30 athymic nude mice. Tumor dimensions were measured using digital calipers (model CD-S6C; Mitutoyo) that have an accuracy of ±0.02 mm. Volume was calculated by the following formula: length × width × depth × 1/2. In the first experiments when the tumors reached a size ~10 mm^3, the mice were randomly divided into four groups of six mice each. Groups of mice were then treated every second day with s.c. injections into the tumor and surrounding regions (a total of seven injections) of HBSS or PA plus LF (0.04, 0.2, and 1 standard doses; 1 standard dose equals 10 μg PA plus 2 μg LF) in 100 μL of HBSS. In subsequent experiments, mice were treated as described above except that treatments with 1 standard dose of inactive or active LeTx began when tumors reached a volume of ~100 mm^3 and a total of six injections was given via the tail vein. Tumor size was monitored before every treatment. After the last injection, mice were euthanized and dissected and then the tumors were either frozen or fixed in 10% neutral-buffered formalin for further analyses.

Immunohistochemistry. Paraffin tissue sections (5 μm) were stained with antimouse antibodies against CD31 (Lab Vision Corp.) and phosphorylated p38 MAPK (Cell Signaling Technology), which were used at a 1:00 dilution. Anti-VEGF (C1; Santa Cruz Biotechnology), VEGF receptor 2 (AF644; R&D Systems), phosphorylated ERK1/2 (9106; Cell Signaling Technology), phosphorylated JNK (Cell Signaling Technology), proliferating cell nuclear antigen (Ab29; Abcam), and apoptosis marker named M30 CytoDEATH (Alexis) were used at a 1:100 dilution. Slides were stained using a Ventana Discovery XT automated immunostainer and a Ventana UltraMap 3,3′-diaminobenzidine detection kit. Photomicrographs of histologic preparations were acquired using a Nikon E600 microscope equipped with a Spot Insight QE camera. For vessel density, we used a Nikon Eclipse 80i with a spectral Nuance camera (CRI). Images were 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 $255\text{-}i_g$, 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 (23) the images produced by the Nuance multispectral imaging system and then applying the connected component (24) to identify contiguous objects (vessels) in the image. Objects <100 square pixels in area were considered background noise and eliminated from the analysis.

Statistical analysis. Values are expressed as mean ± SD except for vascular density, which is ±SE. The difference between pairs of value was evaluated by Student's *t* test and Wilcoxon rank-sum test for vessel quantification. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered as significant.

Results

LeTx cleaves MEK1/2 and inactivates ERK1/2 in SVEC cells. Although LF 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 cells and proteolytically modify MKKs, we treated vGPCR-SVEC cells with LeTx and assayed the NH_2-terminal cleavage of MEK1/2 using antibodies that recognize the NH_2-terminal epitope in uncleaved MEK only (Fig. 1A). Loss of the NH_2-terminal epitope of MEK1/2 was noted in cells that were treated with LeTx but not in cells treated with U0126, DMSO, or PA plus inactive LF. Similar results were obtained with SVEC not expressing vGPCR (data not shown). These results indicate that LF can enter and proteolytically modify MKKs in these cells.

LF-mediated removal of the NH_2-terminal epitope of MKKs blocks MAPK activation (25, 26). To confirm that LeTx prevents MAPK activation in vGPCR-SVEC cells, we probed lysates with antibodies against active ERK1/2, p38 MAPK, and JNK. We found that, whereas levels of ERK1/2, p38 MAPK, and JNK remained constant in all treatments, levels of phosphorylated ERK1/2 and p38 MAPK decreased in response to treatment with LeTx (Fig. 1A). Although only modest phosphorylation of JNK was detected in untreated vGPCR-SVEC, it too was diminished in the presence of LeTx. In contrast, levels of phosphorylated ERK5, a substrate for MEK5, were not diminished in the presence of LeTx. These results are consistent with published observations that LF cleaves MKK1, MKK2, MKK3, MKK4, MKK6, and MKK7 but not MEK5. U0126, a small-molecule

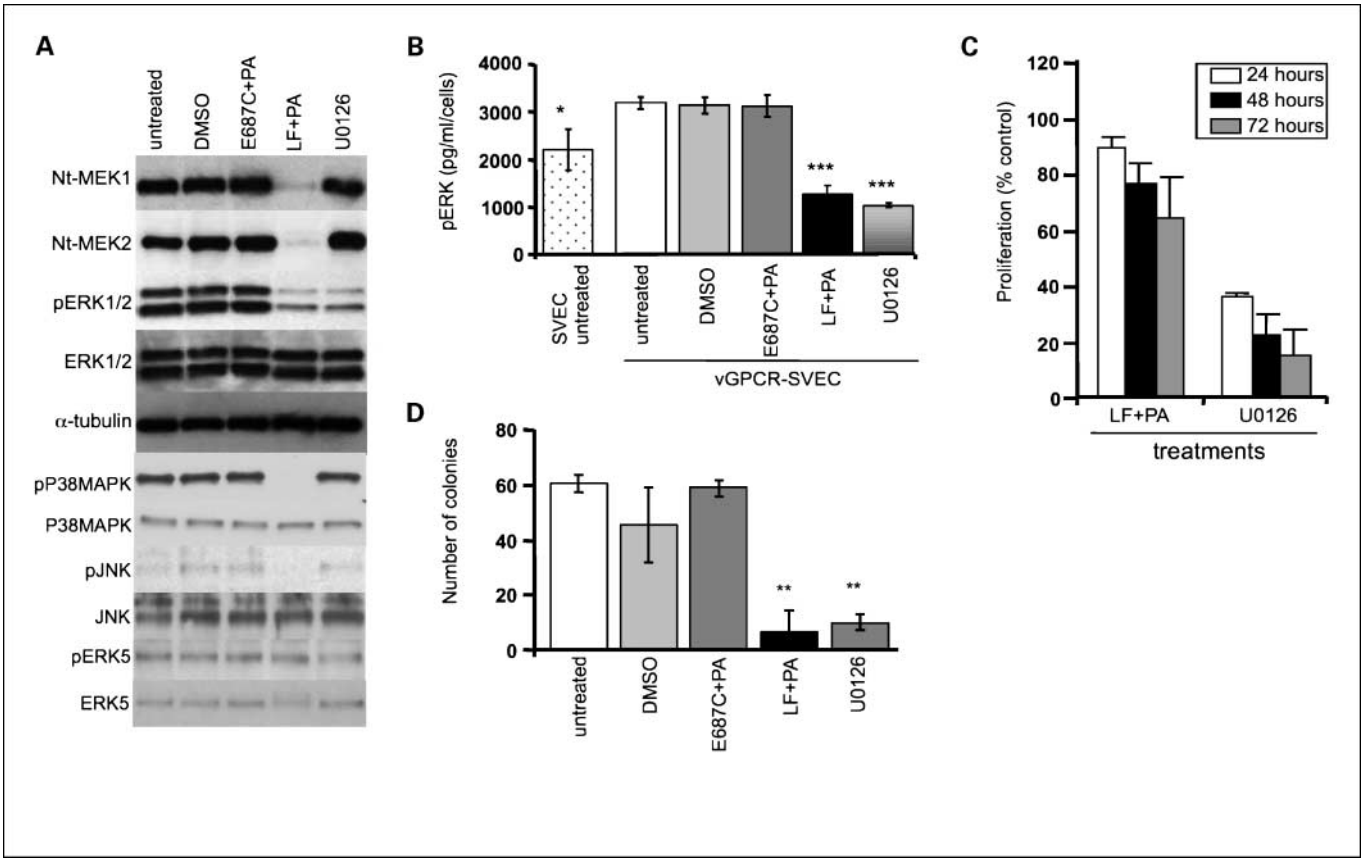

Fig. 1. Effect of LeTx on MEK1/2 pathway. Cells lysates were split for immunoblots and ELISA. Immunoblots (*A*) of vGPCR-SVEC cell lysates show loss of the NH_2-terminal epitope of MEK1 and MEK2 after treatment with LeTx (100 ng/mL LF + 1 μg/mL PA) but not in cells treated with either medium alone (untreated), U0126 (inhibitor of MEK1/2), DMSO (negative control for U0126), or E687C (inactive form of LF) plus PA. Treatment of cells with LeTx caused a decrease of phosphorylated ERK (*pERK1/2*), phosphorylated p38 MAPK (*pP38MAPK*), and phosphorylated JNK (*pJNK*) without effect on total MAPK levels. LeTx did not affect levels of ERK5 phosphorylation (*pERK5*). α-Tubulin was used as a standardization control. ELISA assays (*B*) of SVEC parental cells and vGPCR-SVEC cells showed a higher level (2.5-fold) of phosphorylated ERK in cells expressing vGPCR compared with parental cells and a significant decrease of phosphorylated ERK in cells treated with LeTx or U0126. Neither medium alone, DMSO, nor E687C plus PA had any effects. Results are expressed in picograms of phosphorylated ERK per milliliter normalized to the number of cells and represent at least three independent experiments. *C,* cells were treated with LeTx (100 ng/mL LF + 1 μg/mL PA) or U0126 (10 μmol/L) for 24, 48, and 72 h. Results represent three independent experiments normalized to untreated cells. *D,* anchorage-independent growth of vGPCR-SVEC cells was evaluated by soft agar colony formation. Columns, mean of three independent experiments; bars, SD. Statistical significance was determined using the Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

MEK1/2 inhibitor, decreased levels of ERK1/2 phosphorylation but did not affect p38 MAPK or JNK activities. Cells treated with inactive LF or DMSO showed no change in levels of phosphorylated MAPK.

We also quantified changes in ERK signaling using an ELISA kit. Consistent with previous observations, expression of vGPCR in SVEC cells caused an increase in ERK signaling above levels observed in nontransfected SVEC (Fig. 1B). Treatment of vGPCR-SVEC cells with either LeTx or U0126 caused a >50% reduction in the levels of active ERK1/2. These results indicate that LeTx can inhibit MKK signaling in these cells.

LeTx modestly decreases cell proliferation. LeTx treatment of Ras-transformed NIH3T3 cells with LeTx causes a modest (~10%) reduction in cell proliferation (4). In contrast, LeTx causes an abrupt lytic death of certain strains of macrophage-derived cell lines (27) and melanoma cells are induced to undergo G_1-S arrest and subsequent apoptosis (5). To determine whether LeTx affects the proliferation of vGPCR-SVEC cells, we treated cells with PA (1 μg/mL) plus various concentrations of LF (0-10,000 ng/mL) for 24, 48, and 72 h. At concentrations in excess of 10 ng/mL, LF caused a moderate but significant (25%) decrease in vGPCR-SVEC proliferation (Fig. 1C; Supplementary Data). In contrast, although U0126 inhibited ERK activation to a similar degree, 10 μmol/L U0126 caused a substantial (60-80%) decrease in vGPCR-SVEC proliferation over the same time period (Fig. 1C). DMSO did not affect cell proliferation (data not shown). These data indicate that LeTx has only a moderate effect on the proliferative capacity of vGPCR-SVEC cells.

LeTx inhibits anchorage-independent growth. Studies with Ras-transformed NIH3T3 cells showed that LeTx blocked the ability of these cells to grow in soft agar (4). To determine whether vGPCR-SVEC cells were able to grow in an anchorage-independent fashion and whether that ability required MKK signaling, we did soft agar colony formation assays in the presence or absence of LeTx. Cells were incubated for 2 weeks after which we quantified the formation of colonies (>500 μm in diameter; Fig. 1D). SVEC parental cells suspended in soft agar failed to proliferate and remained as single cells in suspension (data not shown). By contrast, vGPCR-SVEC cells proliferated and formed colonies. Treatment of vGPCR-SVEC cells with LeTx decreased colony formation. A similar effect was observed with 10 μmol/L U0126. However, because U0126 had a profound effect on proliferation (Fig. 1C), it is not clear that the failure to form colonies had anything to do with their ability to grow in an anchorage-independent fashion. Neither inactive LF nor DMSO significantly altered colony formation. These results indicate that the expression of vGPCR in SVEC cells supports the ability of these cells to grow in an anchorage-independent fashion and requires MKK signaling.

LeTx decreases VEGF release. vGPCR has been shown to induce VEGF release from NIH3T3 cells (19) and to modulate expression of cytokines in endothelial cells (20). To determine whether expression of vGPCR in SVEC cells induces VEGF release in a MKK-dependent fashion, we measured VEGF in conditioned medium from cells treated with or without LeTx or U0126. Consistent with published reports, expression of vGPCR in SVEC cells led to a 3-fold elevation of VEGF release relative to SVEC parental cells (Fig. 2). Interestingly, treatment with LeTx but not U0126 significantly decreased VEGF release

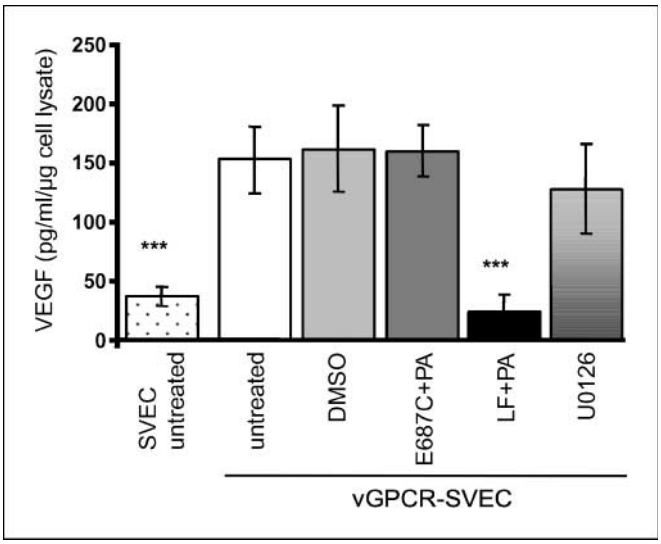

Fig. 2. LeTx decreases VEGF release. Levels of VEGF present in conditioned medium were evaluated using ELISA assay. Columns, VEGF levels (pg/mL) have been normalized to the protein content of the corresponding cell lysate and represent three independent experiments; bars, SD. Statistical significance was determined using the Student's *t* test (***, $P < 0.001$).

from vGPCR-SVEC cells (Fig. 2). This indicates that expression of vGPCR in these cells induced VEGF release in a MKK-dependent fashion.

To determine whether this inhibition was specific to VEGF rather than a global effect on cytokine release, we independently examined the effects of LeTx on the release of five additional cytokines. Treatment with LeTx caused a moderate but significant reduction in the release of RANTES, interleukin-6, and murine interleukin-8 (KC; Table 1, VEGF release measured in the preceding ELISA is included for comparison). U0126 had a similar although more pronounced effect on the same cytokines. Neither inactive LF nor DMSO had a significant effect on release of these cytokines. In contrast, the release of cytokines, such as stromal cell–derived factor-1β and transforming growth factor-1β, was not influenced by LeTx or U0126 (Table 1). These results indicate that LF impairs the release of specific cytokines, notably VEGF, from vGPCR-SVEC cells.

In vivo *tumorigenicity assays.* To study the effect of LF on tumor development, vGPCR-SVEC cells were injected s.c. into athymic nude mice. In our initial experiments, we did intratumoral injections to verify tumor sensitivity. These treatments began when the tumors reached an approximate volume of 10 mm^3. Mice were injected with either 1 standard dose of PA plus inactive LF (10 μg PA plus 2 μg LF) or 0.04 to 1 standard doses of LeTx. At the end of the experiment (seven injections), we noted that tumors injected with 1 standard dose of LeTx had a significantly reduced volume compared with tumors that had been injected with HBSS (see Supplementary Data). Histologic examinations of sections from these tumors stained with H&E showed an increase of apoptotic and necrotic cells in tumors treated with LeTx relative to HBSS treatment (Supplementary Data).

In subsequent experiments, we evaluated the response of vGPCR-SVEC xenografts to LeTx given systemically by i.v. injection in the tail vein. Groups of nine mice were injected with 1 standard dose of either active or inactive LeTx every

Table 1. Screening of cytokine releases

Cytokine	vGPCR-SVEC treatments			
	DMSO	E687C + PA	LF + PA	U0126
VEGF	105.39 ± 36.28	104.61 ± 22.05	15.59 ± 14.77*	83.76 ± 37.05
RANTES	100.96 ± 11.56	108.45 ± 11.18	77.54 ± 6.09 †	36.47 ± 19.92 †
KC	89.14 ± 3.49	103.87 ± 9.55	69.98 ± 13.35 ‡	52.27 ± 12.58 †
Interleukin-6	89.14 ± 11.33	96.52 ± 12.79	56.98 ± 7.14*	44.14 ± 10.82*
TGF-1β	101.72 ± 19.12	98.14 ± 29.39	101.72 ± 35.57	94.14 ± 15.71
SDF-1β	99.59 ± 17.47	71.34 ± 38.25	96.44 ± 10.37	146.13 ± 56.96

NOTE: Conditioned medium from vGPCR-SVEC cell incubations was screened for cytokines after 24 h of treatment. Results were normalized to cytokines secreted from untreated cells (set to 100%). Values are expressed ± SD and results were evaluated by the Student's *t* test.
Abbreviations: TGF-1β, transforming growth factor-1β; SDF-1β, stromal cell–derived factor-1β.
*$P < 0.001$.
†$P < 0.01$.
‡$P < 0.05$.

second day for 2 weeks (six injections total). Typically, injections started when tumors attained an average volume of ~50 mm^3. As shown in Fig. 3, LeTx treatment caused a significant decrease ($P = 0.02$) in tumor volume when compared with tumors treated with inactive LeTx. Consistent with published observations, gross morphologic analysis revealed this dose of LeTx had no noticeable effect on animal health (4, 6, 7). These observations indicate that growth of vGPCR-SVEC xenografts is inhibited by LeTx.

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 specific for active MAPK (Fig. 4) as well as markers of proliferation (proliferating cell nuclear antigen) and cell death (M30 CytoDEATH; Fig. 5). Control tumors treated with PA plus inactive LF contained viable proliferating cells throughout the tumor. In these lesions, regions of active ERK1/2 and p38 MAPK signaling were readily evident. By contrast, comparison of staining in serial sections of LeTx-treated tumors showed focal necrosis and a marked reduction in proliferating (proliferating cell nuclear antigen positive) cells. In addition, regions of ERK1/2 and p38 MAPK activity were reduced in LeTx-treated tumors. Active JNK was not detected in tumor tissues from treated or control animals, although it was detected in adjacent normal tissues (data not shown). These results indicate that the robust growth of vGPCR-SVEC xenograft tumors requires MKK signaling.

Because the growth of this tumor is thought to be promoted by the release of angioproliferative cytokines and because we have observed that LF has a pronounced effect on VEGF release from vGPCR-SVEC, we also examined fixed tumors for the presence of VEGF and its receptor, VEGF receptor 2. Immunostaining documented the levels of each were significantly reduced after LeTx treatment when compared with tumors that had been treated with inactive LeTx (Fig. 5). As an independent confirmation, we also compared the staining of CD31, an endothelial marker. Similar to VEGF, staining with CD31 was noticeably decreased in LeTx-treated tumors (Fig. 6A and B). Moreover, the blood vessels in toxin-treated tumors seemed to have been disrupted (Fig. 6C and D). Quantification of randomly chosen fields within these images shows that LeTx caused a reduction in vascular density >80% (Fig. 6E). These results indicate that LeTx reduces the release of angioproliferative cytokines from vGPCR-SVEC xenografts and disrupts vascular patterning within these tumors.

Discussion

We initiated this study to test the hypothesis that inhibition of MKK signaling is an effective strategy for treatment of Kaposi's sarcoma using LeTx and vGPCR-expressing mouse endothelial cells as the experimental model (12). We now believe that the use of LeTx may indeed be an effective strategy for blocking the release of angioproliferative factors that drive Kaposi's sarcoma growth. Several independent lines of evidence

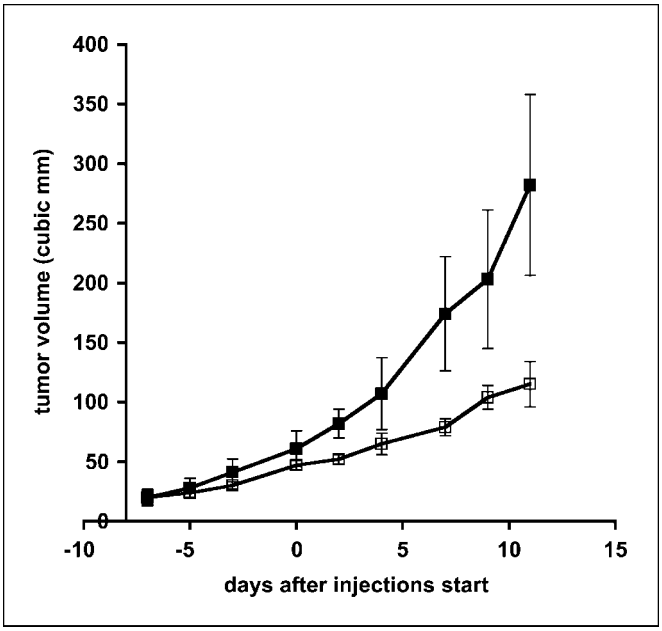

Fig. 3. Systemic treatment by LeTx. vGPCR-SVEC cells were injected s.c. into the dorsal flank of 20 athymic nude mice. When tumors reached an average volume of 50 mm^3, mice were divided randomly into two groups that were treated by tail vein injection with either 2 μg E687C + 10 μg PA (■) or 2 μg LF + 10 μg PA (□) every second day for a total of six injections. Tumors were measured before each injection. Statistical significance between tumors treated with LeTx versus PA + E687C was determined using the Student's *t* test ($P < 0.02$).

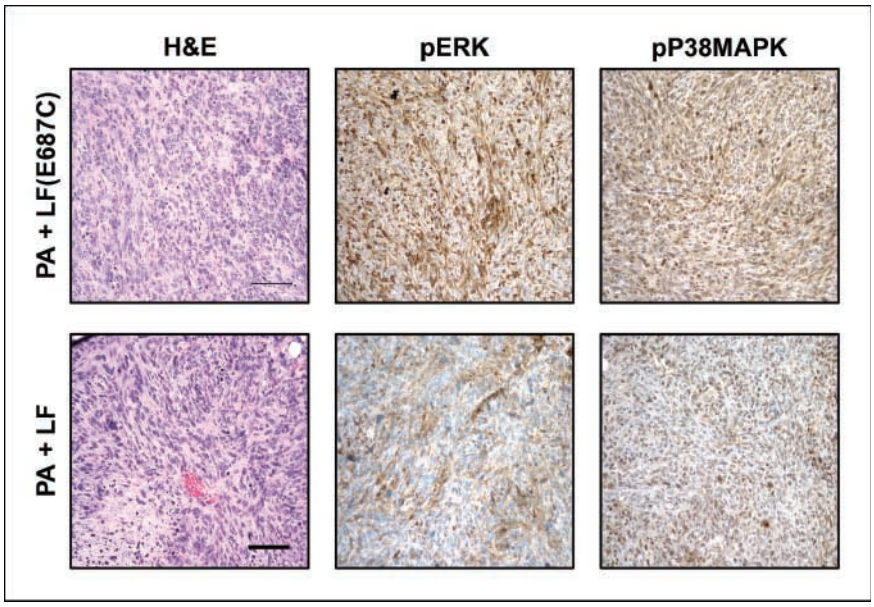

Fig. 4. LeTx causes focal injury and decreased MAPK activity. Tumors treated with either PA plus inactive LF (*top*) or PA plus LF (*bottom*) were stained for H&E, ERK1/2 phosphorylation (*pERK1/2*), or p38 MAPK phosphorylation (*pP38MAPK*). LeTx-treated tumors showed evidence of focal necrosis and decreased MAPK phosphorylation. All images were acquired with a Nikon E600 microscope at ×20 magnification. Bar, 100 μm.

support this conclusion. Although LeTx has only a modest effect on the proliferative capacity of vGPCR-SVEC, it substantially reduces VEGF release from these cells and prevents their anchorage-independent growth. Moreover, LeTx significantly reduces tumor levels of VEGF and VEGF receptor 2, disrupts tumor vasculature, and reduces the growth of vGPCR-SVEC xenografts *in vivo*. To explain these results, we propose that inhibition of MKK signaling by LeTx blocks the ability of these cells to release angioproliferative cytokines that act in both an autocrine fashion (as shown in soft agar colony assays) and a paracrine fashion (as shown in xenografts) to promote tumor growth.

Besides LeTx, which inhibits MKK1 to MKK7 (with the exception of MKK5), other MKK inhibitors may have a similar effect on Kaposi's sarcoma; indeed, most of the effects we observed for LeTx were also noted with U0126. However, the response of vGPCR-SVEC cells to U0126 differed from the response to LeTx in two key aspects. First, despite the fact that both LeTx and U0126 inhibited ERK1/2 activity to a similar degree, LeTx had only a modest effect on proliferation, whereas U0126 profoundly inhibited proliferation. We have made similar observations in fibrosarcoma cells (4). One possible explanation for this discrepancy is that inhibition of another U0126 target, such as MEK5 (28, 29), mediates cell cycle arrest. However, this seems unlikely because we did not observe a decrease in ERK5 phosphorylation following treatment by U1026. An alternative explanation that we favor is that the activity of a MKK besides MEK1 or MEK2 is required to respond to U0126 in a cytostatic fashion. Support for this possibility comes from recent observations in endothelial cells that the decision either to proliferate or become motile is coordinately regulated by a balance of ERK and p38 MAPK activities (30), respectively. In this case, as we observed for U0126 treatment, in the absence of ERK activity, p38 MAPK would be expected to induce a cell cycle arrest. However, in the absence of both ERK and p38 MAPK activity, as occurs with LeTx, cell cycle arrest

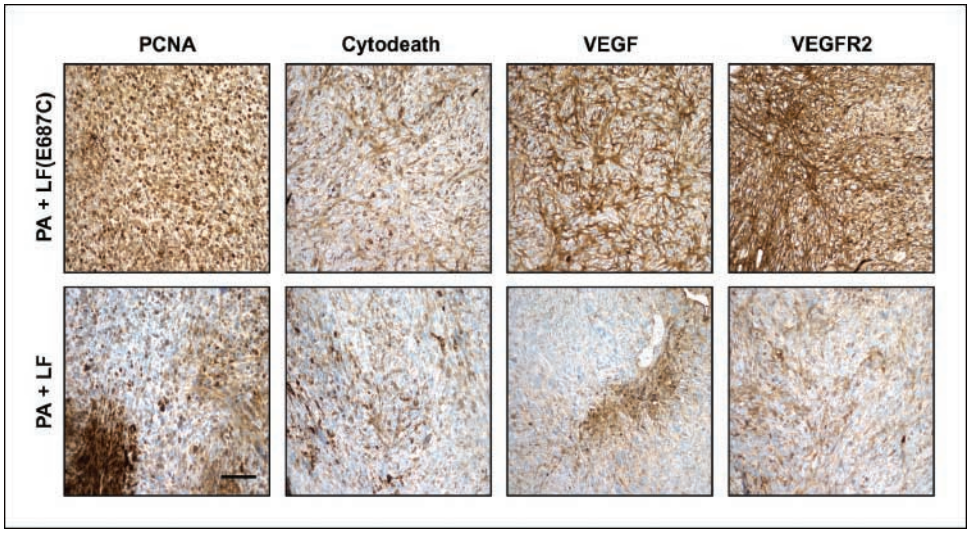

Fig. 5. LeTx decreases tumor cell proliferation and vascular signaling. Tumors treated with either PA plus inactive LF (*top*) or PA plus LF (*bottom*) were immunostained for markers of proliferation [proliferating cell nuclear antigen (*PCNA*)], cell death (*Cytodeath*), and vascular signaling [*VEGF* and VEGF receptor 2 (*VEGFR2*)]. LeTx-treated tumors showed evidence of decreased proliferation and vascular signaling. All images were acquired with a Nikon E600 microscope at ×20 magnification. Bar, 100 μm.

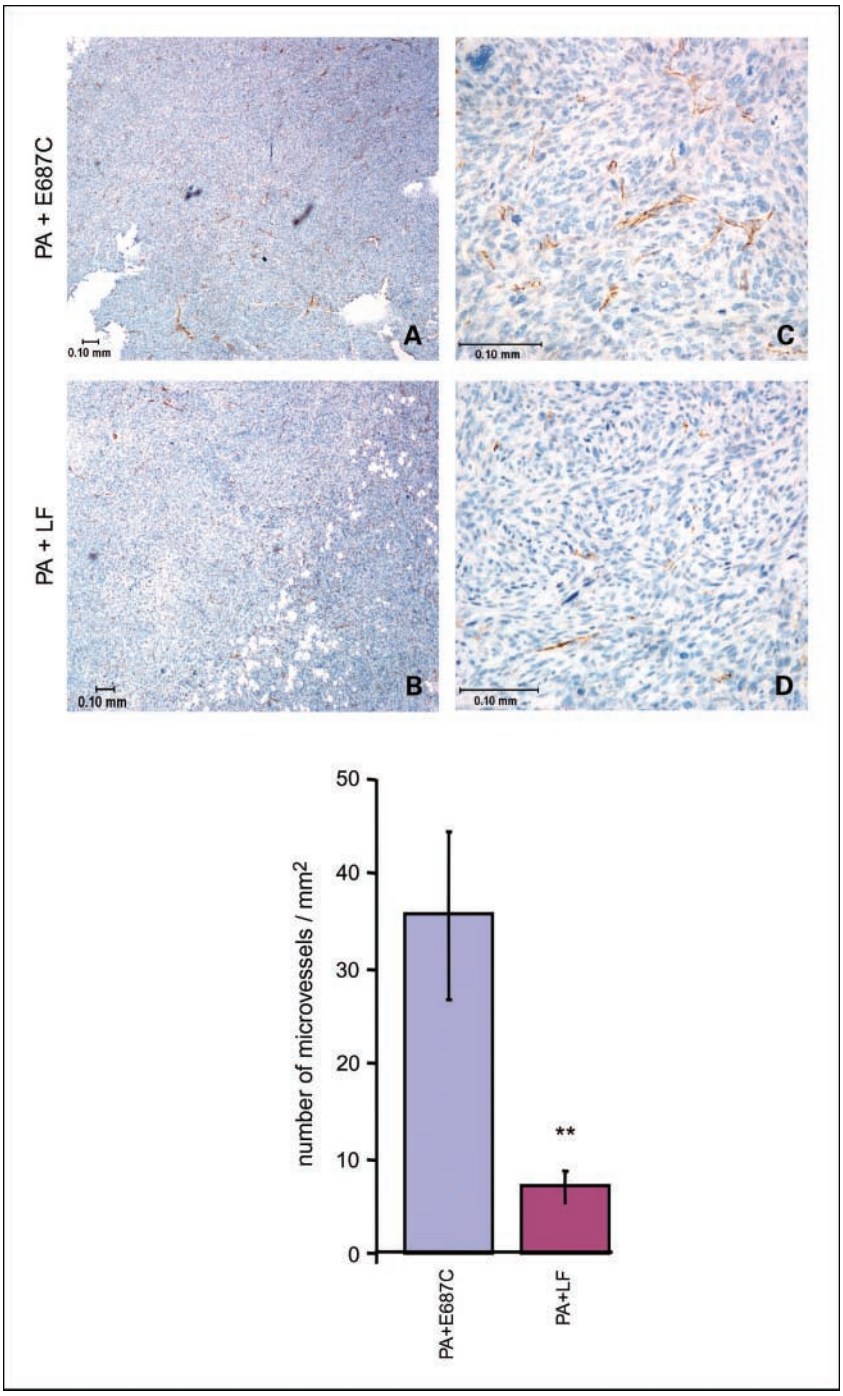

Fig. 6. LeTx decreases microvessel density. *A* to *D*, immunohistochemical analysis of CD31 staining after 2 wk of treatment at ×4 (*A* and *B*) and ×20 (*C* and *D*) magnification. Microvessel density (*right*) was quantified using three images from each of four different tumors per group. Statistical significance was determined using Wilcoxon rank-sum test (**, $P < 0.01$). Bar, 100 μm.

would not occur. Second, only LeTx had a strong effect on the release of VEGF. This observation indicates that U0126-mediated inhibition of MEK1/2 is not sufficient to block VEGF release from vGPCR-SVEC and that other MKKs may play a role in its release. It should be noted, however, that in other cell types we have tested, including melanoma- and fibrosarcoma-derived cell lines, U0126 does cause a decrease in VEGF release,[4] although not to the same extent as LeTx. Similarly, Akula et al. (31) showed that 1 h of treatment of HHV8-infected B cells with 50 μmol/L PD98059, another MEK1/2 inhibitor (32), causes a substantial decrease in levels of VEGF mRNA and protein. These observations indicate that, although MKK signaling is an important regulator of VEGF release, some aspects of its control may be cell type specific.

We noted that LeTx reduced not only VEGF release but also the release of other angioproliferative cytokines, including RANTES and interleukin-6. Moreover, Ye et al. (33) recently showed that signaling through multiple MKK pathways regulates angiopoietin-2 expression in HHV8-infected endothelial cells. This

[4] Young J, Ding Y, Duesbery N, unpublished data.

indicates that MKK pathways influence the release of several cytokines that promote angiogenesis. Kaposi's sarcoma is particularly fascinating to study in this regard because it is the release of angioproliferative factors from the spindle cells that drives its growth (14–17). However, many tumor cell types have been shown to release factors, such as VEGF and interleukin-8, as they co-opt host endothelial cells to supply blood to the tumor (34–37). Indeed, we have noted that LeTx also inhibits the release of angioproliferative cytokines from other cell types, including fibrosarcoma cells.[4] Thus, inhibition of MKKs by LeTx or a similar agent may be a generally effective strategy for reducing cytokine-driven tumor growth and vascularization.

Potential obstacles to the use of LeTx in the clinic exist. First, PA and LF are immunogenic and will elicit an adaptive immune response in patients. However, current PA-based anthrax vaccines that are given in the presence of adjuvant by s.c. or i.m. routes typically do not cause a significant anti-PA response until 30 to 40 days after vaccination. Indeed, a recent study of antibody response in human volunteers injected with anthrax vaccine absorbed (AVA, BioThrax) found that the mean time to seroconversion was ~27.7 days (range, 14-63 days; ref. 38). Moreover, it is apparent that the presence of adjuvant and formaldehyde in that vaccine contributes significantly to this response (39). Thus, it is conceivable that LeTx given in the absence of adjuvant may be used as a therapeutic agent within this time interval. In addition, whereas strains of some species (e.g., rats) exhibit acute sensitivity to LeTx (40), the relative sensitivity of humans to the highly purified, clinical-grade PA and LF used in this study is not certain. Therefore, careful preclinical toxicologic testing of LeTx is warranted.

Finally, it is worth noting that MAPKs were originally identified as key members of signal transduction pathways that transmit regulatory cues from the extracellular environment to the cytoplasm and nucleus where they affect changes in cellular activities (41). However, the data we and others have presented now show that in tumor cells the reverse is also true. MKKs transmit signals originating within the cell and moving to the extracellular environment to do regulatory functions. In the case of tumors, MKKs play an essential role in the release of angioproliferative cytokines that promote their growth and vascularization.

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