BRAF status and mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin

Ralph J. Abi-Habib,[1] Jeffrey O. Urieto,[2] Shihui Liu,[3] Stephen H. Leppla,[3] Nicholas S. Duesbery,[4] and Arthur E. Frankel[2]

Departments of [1]Biochemistry and Molecular Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [2]Hematology/Oncology and Cancer Research Institute, Scott & White Hospital, Temple, Texas; [3]Microbial Pathogenesis Section, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland; and [4]Van Andel Research Institute, Grand Rapids, Michigan

Abstract

Anthrax lethal toxin, composed of protective antigen and lethal factor, was tested for cytotoxicity to human melanoma cell lines and normal human cells. Eleven of 18 melanoma cell lines were sensitive to anthrax lethal toxin (IC_{50} < 400 pmol/L) and 10 of these 11 sensitive cell lines carried the V599E BRAF mutation. Most normal cell types (10 of 15) were not sensitive to anthrax lethal toxin and only 5 of 15 normal human cell types were sensitive to anthrax lethal toxin (IC_{50} < 400 pmol/L). These cells included monocytes and a subset of endothelial cells. In both melanoma cell lines and normal cells, anthrax toxin receptor expression levels did not correlate with anthrax lethal toxin cytotoxicity. Furthermore, an anthrax toxin receptor–deficient cell line (PR230) did not show any enhanced sensitivity to anthrax lethal toxin when transfected with anthrax toxin receptor. Anthrax lethal toxin toxicity correlated with elevated phosphorylation levels of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) 1/2 in both melanoma cell lines and normal cells. Anthrax lethal toxin–sensitive melanoma cell lines and normal cells had higher phospho-MEK1/2 levels than anthrax lethal toxin–resistant melanoma cell lines and normal tissue types. U0126, a specific MEK1/2 inhibitor, was not toxic to anthrax lethal toxin–resistant melanoma cell lines but was toxic to 8 of 11 anthrax lethal toxin–sensitive cell lines. These results show that anthrax lethal toxin toxicity correlates with elevated levels of active MEK1/2 pathway but not with anthrax toxin receptor expression levels in both normal and malignant tissues. Anthrax lethal toxin may be a useful therapeutic for melanoma patients, especially those carrying the V599E BRAF mutation with constitutive activation of the mitogen-activated protein kinase pathway. [Mol Cancer Ther 2005;4(9):1303–10]

Received 5/9/05; revised 6/26/05; accepted 7/8/05.

Grant support: Van Andel Research Institute (Grand Rapids, MI).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Arthur E. Frankel, Scott & White Hospital, 2401 South 31st Street, Temple, TX 76508. Phone: 512-724-0094; Fax: 512-724-0098. E-mail: afrankel@swmail.sw.org

Copyright © 2005 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0145

Introduction

Patients with metastatic melanoma respond poorly to chemotherapy with response rates of 20% and median response duration of 3 months (1). New treatment strategies are needed. The mitogen-activated protein (MAP) kinase (MAPK) pathway is a highly conserved signaling pathway mainly responsible for cell proliferation, survival, and differentiation. Mutations leading to the constitutive activation of the Ras-Raf-MAP/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2)-ERK1/2 MAPK pathway are a hallmark of many human cancers including melanomas. The V599E BRAF mutation has been found in 70% of human melanomas whereas another 20% have been found to carry the Q61K/R N-Ras mutation (2). Both of these mutations lead to the constitutive activation of the MAPK pathway independently of any upstream growth signals and, therefore, to the uncontrolled growth and survival of melanoma cells. Furthermore, several studies have shown that the constitutive activation of the Ras-Raf-MEK1/2-ERK1/2 MAPK pathway is critical for melanoma survival and proliferation (3, 4). Hence, targeting the MAPK pathway is an attractive strategy for melanoma therapy. We recently showed that anthrax lethal toxin proteolytically cleaves MEKs (4). Thus, we hypothesize that anthrax lethal toxin might be a potential treatment for melanoma.

Anthrax lethal toxin is composed of two proteins: protective antigen and lethal factor. Protective antigen binds cell surface receptors tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene-2 (CMG2; refs. 5, 6). After cell binding and furin processing, the 63 kDa protective antigen fragments heptamerize, bind three molecules of lethal factor, migrate to lipid rafts, and undergo endocytosis (7). In acidic endosomes, the complex forms pores through which lethal factor reaches the cytosol and cleaves and inactivates MEKs (4). This leads to cell growth inhibition and cell death.

We have previously shown cytotoxicity of anthrax lethal toxin to melanoma cell lines (8). In this study, we further characterize the specificity, potency, and molecular mechanisms for cytotoxicity on human melanoma cells, normal cells, and transfected cell lines.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

Materials and Methods

Reagents

Both protective antigen and lethal factor were expressed and purified as described previously (9). We also employed a variant of lethal factor (FP59) to induce MAPK--independent, protein synthesis inhibition–mediated cell death. FP59 is a fusion of the protective antigen binding domain of lethal factor (amino acids 1–254) with the ADP-ribosylation domain of *Pseudomonas aeruginosa* exotoxin A (amino acids 362–613). FP59 was expressed and purified as described previously (10). U0126, a specific MEK1/2 inhibitor, was purchased from Cell Signaling Technology (Beverly, MA) and used as indicated.

Cells and Cell Lines

Melanoma cell lines were purchased from the American Type Culture Collection (Manassas, VA) and from the Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (Braunschweig, Germany) and grown as recommended. Normal human cells were purchased from Cambrex (Baltimore, MD) and grown as recommended. PR230, a spontaneous protective antigen receptor–deficient Chinese hamster ovary (CHO) cell mutant, as well as TEM8- and CMG2-transfected CHO cells were generated as described previously (11). TEM8 and CMG2 expression was enriched by four rounds of flow cytometry with phycoerythrin-streptavidin/biotin-protective antigen staining.

Cytotoxicity Assays

[^{3}H]Leucine incorporation inhibition assay and [^{3}H]thyhymidine incorporation inhibition assay were done as described previously (12). In short, aliquots of 10^4 cells were coincubated with 1 nmol/L lethal factor or FP59 in 100 μL medium in Costar 96-well flat-bottomed plates in duplicates. Fifty microliters of protective antigen in medium were added to each column to yield concentrations ranging from 10 to 10,000 pmol/L (for U0126, concentrations ranged from 1 nmol/L to 100 μmol/L), and the cells were incubated at 37°C/5% CO_2 for 48 hours. [^{3}H]Thymidine (1 μCi; NEN DuPont, Boston, MA) in 50 μL medium was added to each well and incubation was continued for an additional 18 hours at 37°C/5% CO_2. For [^{3}H]leucine incorporation inhibition, media was removed and 1 μCi [^{3}H]leucine (NEN DuPont) in 100 μL of leucine-free media was added to each well, and incubation was continued for an additional 18 hours at 37°C/5% CO_2. Cells were then harvested with the Skatron Cell Harvester (Skatron Instruments, Lier, Norway) onto glass fiber mats and counts per minute of incorporated radiolabel were counted using an LKB liquid scintillation counter gated for ^{3}H. The IC_{50} was defined as the concentration of toxin which inhibited thymidine or leucine incorporation by 50% compared with control wells. The percent maximal [^{3}H]thymidine or [^{3}H]leucine incorporation was plotted versus the log of the toxin concentration, and nonlinear regression with a variable slope sigmoidal dose response curve was generated along with IC_{50} using GraphPad Prism software (GraphPad Software, San Diego, CA).

Anthrax Toxin Receptor Expression Levels

Protective antigen was labeled with Bolton-Hunter ^{125}I as described previously (13). Anthrax toxin receptor expression levels were measured using ^{125}I-labeled protective antigen binding assay as previously described for ^{125}I-labeled epidermal growth factor binding assay with the following differences (14). Cells (10^6) were plated in 12-well plates and incubated with varying amounts of ^{125}I-labeled protective antigen (4,000–5 pmol/L) with or without excess (4 nmol/L) cold protective antigen at 37°C for 1 hour; then the supernatants were removed, the cells washed thrice with PBS containing 2 mmol/L of $CaCl_2$ and $MgCl_2$, and harvested. Experiments were done in duplicate. Receptor number per cell (maximum number of binding sites) as well as dissociation constant (K_d) were calculated with the GraphPad Prism software.

Western Blotting

Cell lysates were obtained and Western blots were done using anti-MEK1/2, phospho-MEK1/2 (Ser217/221), ERK1/2, and phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology) as described previously (15). We controlled equal loading of samples using an anti-β actin monoclonal antibody (Cell Signaling Technology). Band intensity was determined using densitometry and ratios of phosphorylated to total MEK1/2 and ERK1/2 levels were calculated. This method provides only a semiquantitative assessment of MEK1/2 and ERK1/2 activity.

Melanoma Cell Line Mutational Status

The type of BRAF or N-Ras mutations carried by melanoma cell lines was determined using a PCR-single strand conformation polymorphism as previously reported (15–17).

Results

Anthrax Lethal Toxin Cytotoxicity

We tested the cytotoxicity of anthrax lethal toxin on a panel of 18 human melanoma cell lines using a [^{3}H]leucine incorporation inhibition assay. Anthrax lethal toxin was cytotoxic to 11 of 18 human melanoma cell lines ($IC_{50} < 400$ pmol/L) whereas the remaining 7 of 18 cell lines were resistant to anthrax lethal toxin cytotoxicity ($IC_{50} > 750$ pmol/L; Table 1; Fig. 1A).

To determine the *in vitro* specificity of anthrax lethal toxin, we tested its cytotoxicity on a panel of 15 normal human cell types (Table 1; Fig. 1B). Anthrax lethal toxin was not toxic to a majority of these normal cells (10 of 15; $IC_{50} > 750$ pmol/L). Only 5 of 15 normal cells were sensitive to this toxin ($IC_{50} <$ 400 pmol/L), and these were restricted to monocytes and a subtype of endothelial cells.

Anthrax Toxin Receptor Expression Levels

We measured anthrax toxin receptor expression levels in human melanoma cell lines and normal human cells (Table 1). In melanoma cell lines, anthrax toxin receptor expression levels did not correlate with anthrax lethal toxin sensitivity ($P > 0.4$; Fig. 2A). Anthrax toxin receptor levels varied between 2,400 and 39,000 receptors/cell. In anthrax lethal toxin–resistant cell lines, anthrax toxin receptor

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

Table 1. Anthrax lethal toxin–induced cytotoxicity and cell growth inhibition of human melanoma cell lines and normal human cells

Cells and cell lines	Anthrax lethal toxin IC_{50} ([^{3}H]leucine), pmol/L	Anthrax toxin receptor expression (receptors/cell)	Phospho/total MEK1/2 ratio	Mutational status
Human melanoma cell lines				
SK-MEL-28	32	10,000	0.54	V599E BRAF
C32	45	39,000	0.99	V599E BRAF
SK-MEL-31	50	17,000	0.91	Wild-type BRAF
SK-MEL-24	62	23,000	0.3	V599E BRAF
G361	112	7,700	0.75	V599E BRAF
HT144	119	32,000	0.99	V599E BRAF
WM-266-4	139	6,500	0.99	V599E BRAF
M14-MEL	153	16,000	0.27	V599E BRAF
SK-MEL-1	318	10,000	0.99	V599E BRAF
A375	334	3,500	0.22	V599E BRAF
Malme-3M	370	5,800	0.99	V599E BRAF
SK-MEL-5	734	25,000	0.07	Q61K N-Ras
SK-MEL-30	1,058	4,900	0.55	Q61K N-Ras
A2058	1,380	4,300	0.29	V599E BRAF
Mel-Juso	2,245	8,200	0.37	Q61K N-Ras
SK-MEL-3	>10,000	15,000	0.52	Q61K N-Ras
RPMI 7591	>10,000	18,000	0.02	Q61K N-Ras
SK-MEL-2	>10,000	2,400	0.08	Q61K N-Ras
Normal cells				
Renal mesangial cells	4.9	240,000	0.62	
Coronary artery endothelial cells	57	50,000	0.78	
Lung microvascular endothelial cells	80	28,000	0.93	
Monocytes	132	11,000	0.72	
Pulmonary artery endothelial cells	288	8,800	0.68	
Cardiac microvascular endothelial cells	805	2,600	0.13	
Umbilical vein endothelial cells	>10,000	11,000	0.16	
Cardiomyocytes	>10,000	32,000	0.14	
Lung fibroblasts	>10,000	3,700	0.23	
Astrocytes	>10,000	660	0.16	
Hepatocytes	>10,000	11,000	0.15	
Renal proximal tubule epithelial cells	>10,000	69,000	0.03	
Renal cortical epithelial cells	>10,000	4,700	0.06	
Skeletal muscle cells	>10,000	9,900	0.07	
Lymphocytes	>10,000	1,200	0.19	

expression levels varied between 2,400 and 25,000 receptors/cell whereas in anthrax lethal toxin–sensitive cell lines, anthrax toxin receptor expression levels varied between 3,500 and 39,000 receptors/cell.

In normal human cells, anthrax toxin receptor expression levels did not correlate with anthrax lethal toxin toxicity either ($P > 0.3$; Fig. 2B). Anthrax toxin receptor expression levels in normal human cell types varied between 660 and 244,000 receptors/cell (Table 1). Anthrax lethal toxin–resistant normal cells had the same range of anthrax toxin receptor expression levels (660–69,000 receptors/cell) as anthrax lethal toxin–sensitive normal cells (8,800–244,000 receptors/cell).

To show that anthrax toxin receptor expression levels do not affect the sensitivity of either melanoma cell lines or normal cells to anthrax lethal toxin, we tested the sensitivity of three melanoma cell lines (SK-MEL-2: 2,400 receptors/cell; A375: 3,500 receptors/cell; and SK-MEL-5: 25,000 receptors/cell) as well as two normal cell types (cardiomyocytes: 32,000 receptors/cell; lung fibroblasts: 3,700 receptors/cell) to protective antigen/FP59 using a [^{3}H]thymidine incorporation inhibition assay. FP59 is a fusion of the protective antigen binding domain of lethal factor and the ADP-ribosylation domain of *P. aeruginosa* exotoxin A. Therefore, protective antigen/FP59 toxicity is only dependent on the expression of anthrax toxin receptor on cell surface. The use of this combination allows us to show toxin entry into the cells. Protective antigen/FP59 was cytotoxic to all three melanoma cell lines, with IC_{50} = 809, 61, and 35 pmol/L for SK-MEL-2, A375, and SK-MEL-5, respectively, as well as to both normal human cell types, with IC_{50} = 1.2 and 1.4 pmol/L for cardiomyocytes and lung fibroblasts, respectively (Table 2; Fig. 2C). These results indicate that anthrax toxin receptor expression levels, in both anthrax lethal toxin–sensitive and anthrax lethal toxin–resistant melanoma cell lines and normal cells, allow for toxin entry into the cells. Therefore, anthrax lethal toxin cytotoxicity is independent of anthrax toxin receptor expression levels.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

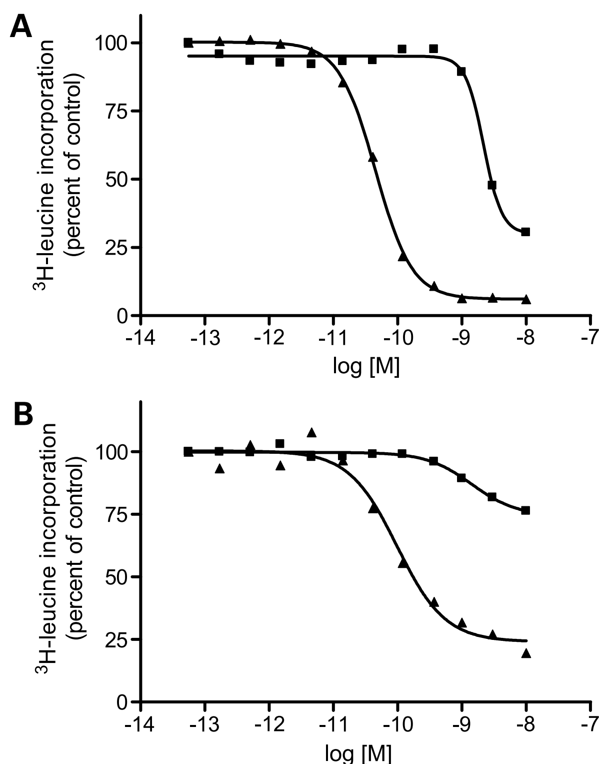

Figure 1. Anthrax lethal toxin cytotoxicity to two human melanoma cell lines, C32 and Mel-Juso (**A**), and to normal human cells, lung microvascular endothelial cells and renal cortical epithelial cells (**B**), using a [^{3}H]leucine incorporation inhibition assay. *X axis,* log of the molar protective antigen concentration; *Y axis,* cell viability expressed as percent control of [^{3}H]leucine incorporation in counts per minute. Lethal factor concentration is 1 nmol/L and is constant all across the plate. C32 (▲) was sensitive to anthrax lethal toxin (IC_{50} = 45 pmol/L) whereas Mel-Juso (■) was resistant to anthrax lethal toxin (IC_{50} = 2,172 pmol/L; **A**). Lung microvascular endothelial cells (▲) were sensitive to anthrax lethal toxin (IC_{50} = 80 pmol/L) whereas renal cortical epithelial cells (■) were resistant to anthrax lethal toxin (IC_{50} > 10,000 pmol/L; **B**).

To further show that anthrax lethal toxin toxicity is independent from anthrax toxin receptor expression levels, we transfected a spontaneous protective antigen receptor–deficient CHO mutant cell line (PR230) with protective antigen receptors TEM8 (TEM-8-PR230) and CMG2 (CMG2-PR230). We then tested the sensitivity of these protective antigen receptor–transfected CHO cells to anthrax lethal toxin and protective antigen/FP59. The PR230 cell line with 530 receptors/cell was not sensitive to anthrax lethal toxin (IC_{50} = 4 nmol/L). Similarly, the protective antigen receptor–transfected TEM8-PR230 and CMG2-PR230 cells with 11,000 and 12,000 receptors/cell, respectively, were not sensitive to anthrax lethal toxin (IC_{50} = 1.6 and 2.4 nmol/L, respectively). However, unlike PR230, TEM8-PR230 and CMG2-PR230 were sensitive to protective antigen/FP59 (IC_{50} = 2,450, 6.6, and 2.7 pmol/L, respectively; data not shown). These results show that overexpression of anthrax toxin receptor on protective antigen–deficient CHO cells induces toxin entry into the cells without affecting the resistance of this cell line to anthrax lethal toxin toxicity. Thus, these results eliminate the possibility of the cell entry step being the anthrax lethal toxin toxicity–determining factor.

MAPK Activation Levels

Because lethal factor cleaves MEKs and inhibits the MAPK pathway and because most melanoma cell lines

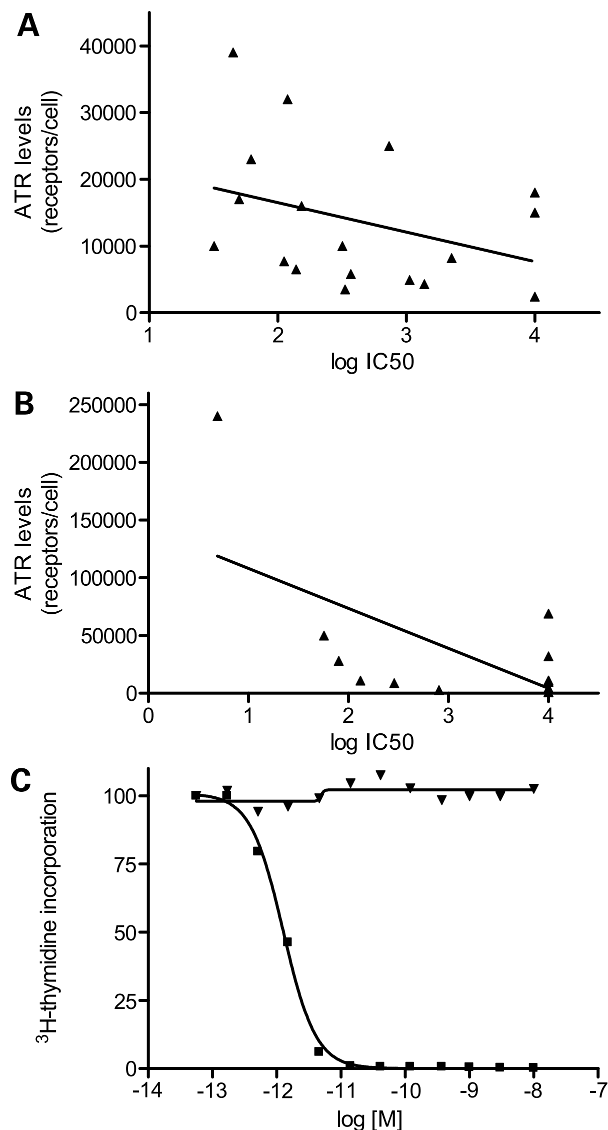

Figure 2. Correlations between anthrax toxin receptor expression levels and IC_{50} of anthrax lethal toxin in melanoma cell lines (**A**) and normal human cells (**B**). *X axis,* log of the IC_{50} of anthrax lethal toxin to cells; *Y axis,* anthrax toxin receptor expression levels (number of receptors per cell). Anthrax toxin receptor expression levels did not correlate with anthrax lethal toxin sensitivity in both melanoma cell lines (P = 0.15) and normal human cells (P = 0.07). **C,** human cardiomyocyte sensitivity to anthrax lethal toxin (protective antigen/lethal factor) and to protective antigen/FP59 using a [^{3}H]thymidine incorporation inhibition assay. *X axis,* log of the molar protective antigen concentration; *Y axis,* cell viability expressed as percent control of [^{3}H]thymidine incorporation in counts per minute. Lethal factor and FP59 concentration is 1 nmol/L and is constant all across the plate. Human cardiomyocytes were resistant to anthrax lethal toxin (▲; IC_{50} > 10,000 pmol/L) but were highly sensitive to protective antigen/FP59 (■; IC_{50} = 1.2 pmol/L).

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

Table 2. Anthrax lethal toxin cytotoxicity, anthrax toxin receptor expression levels, and protective antigen/FP59 cytotoxicity on three human melanoma cell lines and two normal human cells

Cells	Anthrax toxin receptor IC_{50} ([^{3}H]leucine), pmol/L	Anthrax toxin receptor expression (receptors/cell)	FP59 IC_{50} ([^{3}H]thymidine), pmol/L
SK-MEL-2	>10,000	2,400	809
A375	334	3,500	61
SK-MEL-5	734	25,000	35
Cardiomyocytes	>10,000	32,000	1.2
Lung fibroblasts	>10,000	3,700	1.4

(17 of 18) carry N-Ras or BRAF mutations, we decided to look at the activation status of the Ras-Raf-MEK1/2-ERK1/2 pathway to explain cell sensitivity to anthrax lethal toxin. We therefore calculated phospho/total MEK1/2 and phospho/total ERK1/2 ratios in melanoma cell lines, normal human cells, and PR230, TEM8-transfected, and CMG2-transfected CHO cells using Western blots and densitometry. In melanoma cell lines, the mean phospho/total MEK1/2 ratio of anthrax lethal toxin–sensitive lines (0.72 ± 0.19) was significantly higher than that of anthrax lethal toxin–resistant cell lines (0.27 ± 0.22; P = 0.0058). Furthermore, phospho/total MEK1/2 ratios correlated with anthrax lethal toxin sensitivity of melanoma cell lines (P = 0.0079; Fig. 3A). Phospho-ERK1/2 levels, however, did not correlate with anthrax lethal toxin sensitivity. In fact, four of seven anthrax lethal toxin–resistant melanoma cell lines that showed very low levels of phospho-MEK1/2 had very high phospho/total ERK1/2 ratios comparable to those of anthrax lethal toxin–sensitive melanoma cell lines. In normal human cell types, the mean phospho/total MEK1/2 ratio of anthrax lethal toxin–sensitive normal cells (0.75 ± 0.13) was significantly higher than that of anthrax lethal toxin–resistant normal cells (0.11 ± 0.06; P = 0.0001). Furthermore, phospho/total MEK1/2 ratios strongly correlated with anthrax lethal toxin toxicity to normal cells (P = 0.0001; Fig. 3B). Phospho-ERK1/2 levels, on the other hand, did not correlate with anthrax lethal toxin sensitivity of normal cells (data not shown). CHO cell lines had low MEK1/2-ERK1/2 activation levels. These results indicate that anthrax lethal toxin toxicity to both melanoma cell lines and normal cells is dependent on MEK1/2 activation levels.

In addition to MEK1/2, anthrax lethal toxin also inhibits MEK 3, 4, 6, and 7, leading to the inhibition of all three branches of the MAPK pathway. It is possible, therefore, that anthrax lethal toxin toxicity is due to the inhibition of all three branches of the MAPK pathway and not only of the Ras-Raf-MEK1/2-ERK1/2 pathway, although most melanoma cells carry mutations in this pathway. We therefore tested the cytotoxicity of a specific MEK1/2 inhibitor, U0126, on melanoma cell lines using a [^{3}H]leucine incorporation inhibition assay. All anthrax lethal toxin–resistant melanoma cell lines were also resistant to U0126 (IC_{50} > 400 μmol/L). This confirms that these cells do not rely on the MAPK pathway for survival and, therefore, are resistant to the specific inhibition of MEK1/2 as well as to the inhibition of the entire MAPK pathway. On the other hand, 8 of 11 anthrax lethal toxin–sensitive melanoma cell lines were also sensitive to U0126 (IC_{50} < 45 μmol/L), whereas the remaining three cell lines were not sensitive to

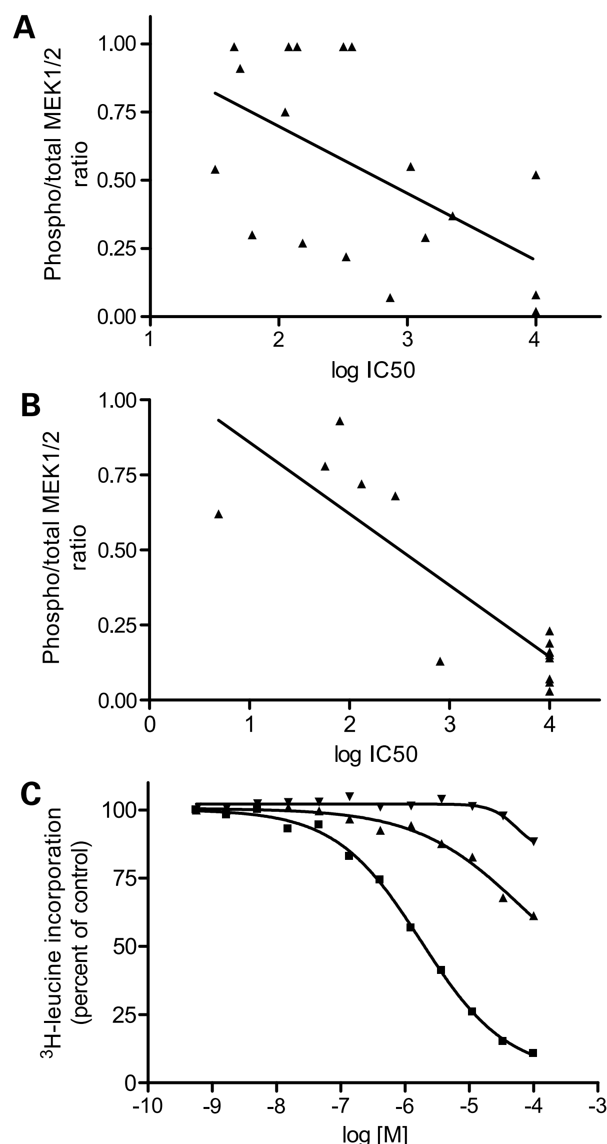

Figure 3. Correlations between phospho/total MEK1/2 ratios and IC_{50} of anthrax lethal toxin in melanoma cell lines (**A**) and normal human cells (**B**). *X axis,* log of IC_{50}; *Y axis,* phospho-total MEK1/2 ratios. Phospho/total MEK1/2 ratios correlated strongly with anthrax lethal toxin cytotoxicity in both melanoma cell lines and normal human cells (P = 0.0079 and 0.0001, respectively). **C,** U0126 toxicity to melanoma cell lines Malme-3M (■), HT144 (▲), and A375 (▼) using a [^{3}H]leucine incorporation inhibition assay. *X axis,* log of the molar protective antigen concentration; *Y axis,* cell viability expressed as percent control of [^{3}H]leucine incorporation in counts per min. Although all three cell lines carry the V599E BRAF mutation and are sensitive to anthrax lethal toxin (IC_{50} = 370, 119, and 334 pmol/L, respectively), only Malme-3M is sensitive to U0126 (IC_{50} = 1.8 μmol/L), whereas HT144 (IC_{50} = 400 μmol/L) and A375 (IC_{50} > 4,000 μmol/L) are resistant to U0126 toxicity.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

Table 3. Anthrax lethal toxin cytotoxicity, phospho/total MEK1/2 ratio, and U0126 toxicity on human melanoma cell lines

Cell lines	LeTx IC_{50} ([^{3}H]leucine), pmol/L	Phospho/total MEK1/2 ratio	U0126 IC_{50} ([^{3}H]leucine), μmol/L
SK-MEL-28	32	0.54	18.6
C32	45	0.99	7.4
SK-MEL-31	50	0.91	8.0
SK-MEL-24	62	0.3	43.5
G361	112	0.75	41.2
HT144	119	0.99	400
WM-266-4	139	0.99	700
M14-MEL	153	0.27	30.4
SK-MEL-1	318	0.99	5.2
A375	334	0.22	>4,000
Malme-3M	370	0.99	1.8
SK-MEL-5	734	0.07	>4,000
SK-MEL-30	1,058	0.55	>4,000
A2058	1,380	0.29	>4,000
Mel-Juso	2,245	0.37	>4,000
SK-MEL-3	>10,000	0.52	433
RPMI 7591	>10,000	0.02	>4,000
SK-MEL-2	>10,000	0.08	452

Abbreviation: LeTx, anthrax lethal toxin.

U0126 (IC_{50} > 400 μmol/L; Table 3; Fig. 3C). This shows that in most melanoma cell lines, anthrax lethal toxin toxicity is due to the inhibition of the Ras-Raf-MEK1/2-ERK1/2 pathway. These results, therefore, confirm the correlation of anthrax lethal toxin toxicity with phospho/total MEK1/2 ratios. However, 3 of 11 anthrax lethal toxin–sensitive melanoma cell lines with high MEK1/2 activation levels were not sensitive to the specific inhibition of MEK1/2 by U0126. In some melanoma cell lines, therefore, anthrax lethal toxin cytotoxicity is due to the inhibition of more than just the MEK1/2 activity.

Melanoma Cell Line Genotype

We then assessed the mutational status of BRAF and N-Ras in our panel of melanoma cell lines. Eleven of 18 human melanoma cell lines carried the V599E BRAF mutation, six melanoma cell lines carried the Q61K/R N-Ras mutation, and one melanoma cell line had both wild-type BRAF and N-Ras (Table 1). The V599E BRAF mutation strongly correlated with anthrax lethal toxin sensitivity of melanoma cell lines with 10 of 11 anthrax lethal toxin–sensitive cell lines carrying the V599E BRAF mutation and 0 of 11 anthrax lethal toxin–sensitive cell lines carrying the N-Ras mutation (P < 0.0001). For anthrax lethal toxin–resistant cell lines, one of seven cell lines had the V599E BRAF mutation whereas six of seven had the N-Ras mutation (P < 0.0001). These results show that anthrax lethal toxin is specifically cytotoxic to human melanoma cell lines carrying the V599E BRAF mutation.

Discussion

In this study, we show that anthrax lethal toxin is highly cytotoxic to a majority of melanoma cell lines (61% of tested cell lines). This confirms and expands findings from previous studies looking at the cytotoxicity of anthrax lethal toxin to eight melanoma cell lines through end-point apoptosis measurement (8). Preliminary studies have shown that anthrax lethal toxin also induces significant cell cycle arrest in more than 50% of melanoma cell lines that are resistant to anthrax lethal toxin cytotoxicity.[5] This is also in accordance with previous studies that describe anthrax lethal toxin–induced cell cycle arrest in certain melanoma cell lines and in some normal cells (8). Furthermore, anthrax lethal toxin is cytotoxic to a restricted number of normal human cells. Only monocytes, renal mesangial cells, coronary artery endothelial cells, pulmonary artery endothelial cells, and lung microvascular endothelial cells were sensitive to anthrax lethal toxin whereas all the other normal cells we tested were resistant. Although we did not test anthrax lethal toxin toxicity to normal human melanocytes in this study, we had already determined in previous work that normal human melanocytes were not sensitive to anthrax lethal toxin. In fact, we did not observe any cell death in human melanocytes incubated with anthrax lethal toxin for up to 192 hours (8). Previous studies had also shown that anthrax lethal toxin is cytotoxic to macrophages and endothelial cells (18, 19). However, this is the first study where 15 different normal human cell types were tested, including six different endothelial cell types. In this study, we therefore confirm that, *in vitro*, anthrax lethal toxin is a potent and relatively selective toxin for the targeting of melanoma cells.

In an attempt to dissect the molecular mechanisms underlying the differential toxicity of anthrax lethal toxin to both human melanoma cell lines and normal human cells, we looked at the first determining step in anthrax lethal toxin toxicity: cell binding. Anthrax toxin receptor expression levels were similar in anthrax lethal toxin–sensitive and anthrax lethal toxin–resistant melanoma cell lines and normal cells and did not correlate with anthrax lethal toxin toxicity. This excludes a determining role of anthrax toxin receptor expression levels in the differential toxicity of anthrax lethal toxin. Furthermore, we showed that toxin cell entry was occurring in both melanoma cell lines and normal cells. Both anthrax lethal toxin–sensitive and anthrax lethal toxin–resistant cells with different anthrax toxin receptor expression levels were sensitive to protective antigen/FP59. Furthermore, we showed that the transfection of protective antigen receptor–deficient PR230 cells with protective antigen receptors TEM8 and CMG2 did not affect resistance to anthrax lethal toxin, although it allowed for toxin entry into the cells. Our observations, therefore, exclude a determining role of anthrax toxin receptor expression levels in the differential toxicity of anthrax lethal toxin.

We then looked at the second determining step in anthrax lethal toxin toxicity: MAPK inhibition. Previous studies

[5] R.J. Abi-Habib et al., unpublished observations.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

have shown that anthrax lethal toxin cytotoxicity is due to the inhibition of the MAPK pathway (20, 21). To determine the contribution of this pathway to cell sensitivity to anthrax lethal toxin, we first determined the mutational status of the Ras-Raf-MEK1/2-ERK1/2 pathway in our panel of melanoma cell lines. We found that 10 of 11 anthrax lethal toxin–sensitive melanoma cell lines carried the V599E BRAF mutation, whereas 6 of 7 anthrax lethal toxin–resistant cell lines carried the Q61K N-Ras mutation. These results indicate that cells carrying the V599E BRAF mutation are much more susceptible to anthrax lethal toxin cytotoxicity than cells carrying the Q61K/R N-Ras mutation. This might be explained by the fact that N-Ras activates other signaling pathways that mediate growth and survival (e.g., phosphatidylinositol 3-kinase/Akt pathway) whereas BRAF does not (22). We then looked at the activation status of the Ras-Raf-MEK1/2-ERK1/2 pathway in both melanoma cell lines and normal cells. Both anthrax lethal toxin–sensitive melanoma cell lines and normal cells had significantly higher levels of phospho/total MEK1/2 ratio as compared with anthrax lethal toxin–resistant melanoma cell lines and normal cells. Furthermore, phospho/total MEK1/2 ratios strongly correlated with sensitivity to anthrax lethal toxin in both melanoma cell lines and normal cells. These results indicate that the sensitivity of melanoma cell lines and normal cells to anthrax lethal toxin depends on the activation levels of the Ras-Raf-MEK1/2-ERK1/2 pathway in these cells. We could not explain, however, the reason why anthrax lethal toxin–resistant melanoma cell lines carrying the N-Ras mutation had lower phospho/total MEK1/2 levels than anthrax lethal toxin–sensitive melanoma cell lines carrying the BRAF mutation because both mutations cause a constitutive activation of the Ras-Raf-MEK1/2-ERK1/2 pathway. We were also unable to explain why anthrax lethal toxin–resistant melanoma cell lines and normal cells with very low phospho-MEK1/2 levels had elevated phospho-ERK1/2 levels.

Because anthrax lethal toxin inhibits all three branches of the MAPK pathway, we wanted to determine whether anthrax lethal toxin cytotoxicity to melanoma cell lines was due to the exclusive inhibition of the Ras-Raf-MEK1/2-ERK1/2 pathway or whether other branches of the MAPK pathway contributed to this toxicity. As expected, the specific MEK1/2 inhibitor U0126 was not cytotoxic to anthrax lethal toxin–resistant melanoma cell lines. It is unlikely that the specific inhibition of the MEK1/2 pathway would be toxic to cells resistant to the inhibition of the entire MAPK pathway. However, when we tested anthrax lethal toxin–sensitive melanoma cell lines, we found that 8 of 11 of these cell lines also showed some degree of sensitivity to U0126, suggesting that in these cell lines, cytotoxicity may be primarily attributed to the inhibition of the Ras-Raf-MEK1/2-ERK1/2 signaling pathway. However, 3 of 11 anthrax lethal toxin–sensitive melanoma cell lines showing high levels of phospho-MEK1/2 were not sensitive to the specific inhibition of the Ras-Raf-MEK1/2-ERK1/2 pathway by U0126. This suggests that in these cell lines, the toxicity of anthrax lethal toxin is not due to the inhibition of the Ras-Raf-MEK1/2-ERK1/2 pathway but might be due to the inhibition of the other branches of the MAPK pathway or to the inhibition of additional, yet unidentified, targets of anthrax lethal toxin. These findings are consistent with earlier observations in melanomas and are in line with recent studies showing that, in some macrophages, anthrax lethal toxin cytotoxicity is due to the inhibition of the p38 MAPK pathway (8, 23). All our results indicate that anthrax lethal toxin is highly cytotoxic to melanoma cell lines and normal cells that depend on the MAPK pathway for survival. Additionally, we found that the V599E BRAF mutation and MEK1/2 phosphorylation levels are a potentially useful marker for MAPK dependency and, subsequently, anthrax lethal toxin sensitivity in melanoma cell lines. However, MAPK dependency may be more complicated than the presence of elevated phospho-MEK1/2 levels alone. Rare melanoma cell lines (2 of 18) were resistant to anthrax lethal toxin although the phospho/total MEK1/2 ratio was high. Further, CHL melanoma cells transfected with V599E BRAF showed a marked increase in phospho/total MEK1/2 ratio but did not have greater anthrax lethal toxin sensitivity.[5] Studies with HER2 in breast carcinomas likewise show complex relationships between pathway components and cell survival. Downstream signals such as phospho-AKT depend on PTEN levels, in addition to HER2 levels, for trastuzumab sensitivity (24). In our study, MEK1/2 activation levels did not always indicate anthrax lethal toxin sensitivity and cell dependency on MAPK pathway for survival.

Our results show that anthrax lethal toxin is a potent toxin for the *in vitro* targeting of melanoma cell lines. The picomolar range potency of anthrax lethal toxin is similar to that of other toxins that were efficacious in the treatment of tumors, both in animal models and in clinical trials (25). Furthermore, anthrax lethal toxin is a specific toxin because it only targets a limited number of normal tissues that rely on the MAPK pathway for growth and survival. More importantly, we determined an easily identifiable marker for anthrax lethal toxin sensitivity, the V599E BRAF mutation. This will allow us to select for anthrax lethal toxin–sensitive melanomas and therefore increase the therapeutic window of this toxin. All these factors argue for further investigation of anthrax lethal toxin as a treatment for BRAF-mutated melanomas.

References

1. Avril MF, Aamdal S, Grob JJ, et al. Fotemustine compared with dacarbazine in patients with disseminated malignant melanoma: a phase III study. J Clin Oncol 2004;22:1118 – 25.

2. Wellbrock C, Ogilvie L, Hedley D, et al. ^{V599E}B-Raf is an oncogene in melanocytes. Cancer Res 2004;64:2338 – 42.

3. Govindarajan B, Bai X, Cohen C, et al. Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. J Biol Chem 2003;278:9790 – 5.

4. Duesbery NS, Webb CP, Leppla SH, et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 1998;280:734 – 7.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025

5. Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA. Identification of the cellular receptor for anthrax toxin. Nature 2001;414:225 – 9.

6. Scobie HM, Rainey JA, Bradley KA, Young JA. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A 2003;11:5170 – 4.

7. Abrami L, Liu S, Cosson P, Leppla SH, van der Goot FG. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J Cell Biol 2003;160:321 – 8.

8. Koo HM, Van Brocklin M, McWilliams MJ, Leppla SH, Duesbery NS, Vande 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;92:3052 – 7.

9. Ramirez DM, Leppla SH, Schneerson R, Shiloach J. Production, recovery and immunogenicity of the protective antigen from a recombinant strain of Bacillus anthracis. J Ind Microbiol Biotech 2002;28:232 – 8.

10. Liu S, Bugge TH, Leppla SH. Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. J Biol Chem 2001 May 25;276:17976 – 84.

11. Liu S, Leppla SH. Cell surface tumor endothelium marker 8 cytoplasmic tail-independent anthrax toxin binding, proteolytic processing, oligomer formation and internalization. J Biol Chem 2003 Feb 14;278:5227 – 34.

12. Abi-Habib RJ, Liu, S, Bugge TH, Leppla S, Frankel AE. A urokinase activated recombinant diphtheria toxin targeting the granulocyte-macrophage colony-stimulating factor receptor is selectively cytotoxic to human acute myeloid leukemia blasts. Blood 2004 Oct 1;104:2143 – 8.

13. Bolton AE, Hunter WM. The labelling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent. Biochem J 1973 Jul;133:529 – 39.

14. Liu TF, Cohen KA, Ramage JG, Willigham MC, Thorburn AM, Frankel AE. A diphtheria toxin-epidermal growth factor fusion protein is cytotoxic to human glioblastoma multiforme cells. Cancer Res 2003 Apr 15;63:1834 – 7.

15. Tsao H, Goel V, Wu H, Haluska F. Genetic interaction between NRAS and BRAF mutations and *PTEN/MMAC1* inactivation in melanoma. J Invest Dermatol 2004 Feb;122:337 – 41.

16. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002 Jun 27;417:949 – 54.

17. Tanami H, Imoto I, Hirasawa A, et al. Involvment of overexpressed wild-type BRAF in the growth of malignant melanoma cell lines. Oncogene 2004 Nov 18;23:8796 – 804.

18. Kirby JE, Anthrax lethal toxin induces human endothelial cell apoptosis. Infect Immun 2004 Jan;72:430 – 9.

19. Popov SG, Villasmil R, Bernardi J, et al. Effect of Bacillus anthracis lethal toxin on human peripheral blood mononuclear cells. FEBS Lett 2002 Sep 11;527:211 – 5.

20. Bardwell AJ, Abdollahi M, Bardwell L. Anthrax lethal factor-cleavage products of MAPK (mitogen-activated protein kinase) kinases exhibit reduced binding to their cognate MAPKs. Biochem J 2004 Mar 1;378:569 – 77.

21. Chopra AP, Boone SA, Liang X, Duesbery NS. Anthrax lethal factor proteolysis and inactivation of MAPK kinase. J Biol Chem 2003 Mar 14;278:9402 – 6.

22. Li W, Zhu T, Guan KL. Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK. J Biol Chem 2004 Sep 3;279:37398 – 406.

23. Park JM, Greten FR, Li ZW, Karin M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 2002 Sep 20;297:2048 – 51.

24. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004 Aug;6:117 – 27.

25. Frankel AE, Neville DM, Bugge TA, Kreitman RJ, Leppla SH. Immunotoxin therapy of hematologic malignancies. Semin Oncol 2003 Aug;30:545 – 57.

Downloaded from http://aacrjournals.org/mct/article-pdf/4/9/1303/1870842/1303-1310.pdf by guest on 24 April 2025