A urokinase-activated recombinant anthrax toxin is selectively cytotoxic to many human tumor cell types

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Abstract

Urokinase plasminogen activator (uPA) is a tumor-specific protease highly expressed in several types of solid tumors and rarely present on normal cells under physiologic conditions. Due to its high expression on metastatic tumors, several different strategies have been used to target the urokinase system. These have mostly led to tumor growth inhibition rather than tumor regression. A different approach was adopted by replacing the furin activation site on a recombinant anthrax toxin with a urokinase activation site. The resulting toxin, PrAgU2/FP59, was highly potent against tumors both in vitro and in vivo. In this study, we show that PrAgU2/FP59 is toxic to a wide range of tumor cell lines, including non–small cell lung cancer, pancreatic cancer, and basal-like breast cancer cell lines. Of the few cell lines found to be resistant to PrAgU2/FP59, most became sensitive upon addition of exogenous pro-uPA. PrAgU2/FP59 was much less toxic to normal human cells. The potency of PrAgU2/FP59 was dependent on anthrax toxin receptor, uPA receptor, and uPA levels but not on total plasminogen activator inhibitor-1 levels. In this study, we show that PrAgU2/FP59 is a wide-range, highly potent, and highly selective toxin that is capable of specifically targeting uPA-expressing tumor cells, independently of the tissue of origin of these cells.

Furthermore, we identify three molecular markers, anthrax toxin receptor, uPA, and uPA receptor, which can be used as predictors of tumor cell sensitivity to PrAgU2/FP59.

Introduction

A significant number of solid tumors have been shown to overexpress extracellular protease systems, which increase their tissue invasiveness and metastatic potential (1, 2). One of the major proteases overexpressed on such tumors is the urokinase plasminogen activator (uPA; ref. 3). uPA is a serine protease that is secreted in a single-chain inactive form (sc-uPA), which is then cleaved by plasmin into a double-chain active uPA. The uPA receptor (uPAR) binds uPA in both its active and inactive forms and protects it from being inhibited by the plasminogen activator inhibitor-1 (PAI-1). The active uPA/uPAR complex cleaves plasminogen into plasmin, thus leading to extracellular matrix degradation and tissue invasiveness and metastasis (4, 5). The uPA system is generally absent on normal cells and is up-regulated only during certain physiologic processes, such as wound healing and tissue remodeling. Although a number of inhibitors of the uPA/uPAR system have been studied as potential cancer therapies, such inhibitors have proven to be more tumor static than cytotoxic. An approach that kills rather than inhibits the growth of urokinase-expressing cells could provide a more effective therapeutic strategy. We, therefore, took advantage of this common characteristic of solid tumors by specifically targeting a recombinant anthrax toxin (PrAg/FP59) to uPA-expressing tumors.

Anthrax lethal toxin is a binary toxin that consists of two separate proteins — protective antigen (PrAg), the cell binding and translocation moiety, and lethal factor, the catalytic moiety (6). PrAg binds cells through the ubiquitously expressed anthrax toxin receptors (ANTXR), tumor endothelial marker-8, and capillary morphogenesis gene-2 (7). PrAg is then cleaved at the sequence 164RKKR167 by cell surface furin-like proteases leading to the release of a 20 kDa fragment. The resulting receptor-bound 63 kDa PrAg fragments then heptamerize, bind three molecules of lethal factor, migrate to lipid rafts, and undergo endocytosis. A recently identified coreceptor, low-density lipoprotein receptor-related protein 6, has been shown to interact with tumor endothelial marker-8 and capillary morphogenesis gene-2 and mediate toxin-receptor complex internalization (8). In acidic endosomes, PrAg complexes form pores through which lethal factor reaches the cytosol. Lethal factor is a zinc metalloprotease that cleaves and inhibits mitogen-activated protein kinase kinases, leading to cell cycle arrest and cell death (6). We and others have...
previously shown that lethal factor is selectively toxic
to tumor cells carrying the V599E BRAF mutation and is
only potent against a limited number of tumors, thus
the inherent limited range of this toxin (9, 10). By substituting
the zinc metalloprotease domain of lethal factor with the
more potent, protein synthesis inhibitor, *Pseudomonas
aeruginosa* exotoxin A (FP59), a novel, more potent,
broader range, anthrax recombinant toxin (PrAg/FP59)
was made (11).

To specifically target uPA-expressing tumors, the furin
cleavage sequence of PrAg 164RKKR167 was substituted
with a urokinase-specific cleavage sequence 163PGSGRSA169
termed U2 (11, 12). The resulting urokinase-activated
recombinant anthrax toxin PrAgU2/FP59 theoretically
binds to all cells through the ubiquitously expressed
ANTXRs but becomes activated only on tumor cells
expressing an active uPA/uPAR system independently of
the tumor type.

Two recent studies have documented that PrAgU2/FP59
is potent and selective against certain tumors *in vivo*
(13, 14). However, our knowledge remains very limited
concerning both the potential usefulness of this toxin
against a broad range of human cancers of different origins
and the molecular markers that can be used to predict
tumor responsiveness to this urokinase-activated toxin. In
this study, we determine the potency, range, and selectivity
of PrAgU2/FP59 and we identify molecular markers that
can be used as predictors of tumor sensitivity.

**Materials and Methods**

**Toxins**

PrAg, PrAgU2, and FP59 were made as described
previously (11). PrAg and PrAgU2 have a molecular
weight of 83 kDa, whereas the molecular weight of FP59
is 59 kDa. The purity of all three proteins used was >99%.

**Cells and Cell Lines**

All human cancer cell lines were purchased from the
American Tissue Culture Collection (Manassas, VA) and
cultured as recommended. Normal human cells were pur-
chased from Cambrex (Baltimore, MD) or American Tissue
Culture Collection and were cultured as recommended.

**Cytotoxicity Assay**

Cytotoxicity was determined using a [3H]thymidine
incorporation inhibition assay as described previously
(15). Briefly, aliquots of 10^4 cells were incubated with
10^−9 mol/L FP59 in 100 μL medium in Costar (Corning,
NY) 96-well flat-bottomed plates in quadruplicates. Fifty
microliters of wild-type PrAg and urokinase-activated
PrAg (PrAgU2) in medium were added to each column
to yield concentrations ranging from 10^{−8} to 10^{−13} mol/L,
and the cells were then incubated at 37°C/5% CO_2 for
48 hours. Then, 1 μCi (0.037 MBq) of [3H]thymidine (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. Cells were then harvested
with the Skatron Cell Harvester (Skatron Instruments, Lier,
Norway) onto glass fiber mats and cpm of incorporated
radiolabel measured using an LKB-Wallac 1205 Betaplate
liquid scintillation counter (Perkin-Elmer, Gaithersburg,
MD) gated for ^3H. The percentage maximal [3H]thymidine
incorporation was then 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). All assays were done at least
twice with an interassay range of ±30% for IC_{50}. For the
blocking assay, 10 ng/mL of an anti-uPA monoclonal
antibody (American Diagnostica, Stanford, CT) were
coincubated with the cells.

**Total uPA and PAI-1 Levels**

Total uPA and PAI-1 levels were determined in supernatants
from all cell lines using ELISA kits (American Diagnostica).
Supernatants were harvested from flat-bottomed 96-well plates in which 10^4 cells were plated in
150 μL medium and incubated for 48 hours at 37°C/
5% CO_2. This allowed for the determination of total uPA
and PAI-1 levels in the same conditions as those of the
cytotoxicity assay.

**uPAR and ANTXR Expression Levels**

The uPAR and ANTXR expression levels were determined
using a [125I]-ligand-receptor binding assay. [125I]labeled
of the sc-uPA and full-length PrAg were done as
described previously (9, 15). The [125I]sc-uPA and [125I]PrAg
binding assays were done as described previously (9).
Briefly, 10^9 cells were plated in 12-well plates and incubated
with varying amounts of [125I]sc-uPA or [125I]PrAg
(2,000–2.5 pmol/L) with or without excess amounts
(4 nmol/L) of cold sc-uPA or PrAg at 37°C for 1 hour; the
supernatants were then removed, and the cells washed
thrice with PBS and harvested using a mammalian protein
extraction buffer (Pierce, Rockford, IL). Experiments were
done in duplicate. Receptor number per cell (maximum
number of binding sites) as well as dissociation constant
(K_d) were calculated using the GraphPad Prism software.

**Statistical Analysis**

We defined sensitivity as cell lines with an IC_{50}
from PrAgU2/FP59 lower than 40 pmol/L. The effects of uPAR
expression levels and total uPA and PAI-1 levels on cell line
sensitivity to PrAgU2/FP59 were determined using uni-
ivariate logistic regression. Wilcoxon's two-sample test was
used to compare the two groups of cell lines (sensitive
versus nonsensitive) with respect to uPAR, uPA, and
PAI-1. Statistical analyses were done with SAS software
(Version 8.02, SAS Institute, Cary, NC). Statistical compar-
isons were made using a 5% level of significance.

**Results**

**Tumor Cell Line Sensitivity to PrAgU2/FP59**

A panel of different human tumor cells was tested for
sensitivity to PrAgU2/FP59 (Table 1). Cells with an IC_{50}
for PrAgU2/FP59 lower than 40 pmol/L were considered
sensitive. The most sensitive tumor types were non–small
lung cancer and pancreatic cancer (four of five and
three of four sensitive cell lines, respectively; Fig. 1). Colon
cancer and prostate cancer cell lines were also sensitive to PrAgU2/FP59 (two of four sensitive cell lines). Only in breast cancer were a minority of cell lines sensitive to PrAgU2/FP59 (three of seven sensitive cell lines; Table 1). However, closer examination of the breast cancer cell lines revealed that all the sensitive cell lines were basal-like, which suggests high potency of PrAgU2/FP59 on basal-like breast cancer. Non–small cell lung cancer cell lines being the most sensitive, we expanded that panel to include a total of 20 cell lines, 14 of which were sensitive to PrAgU2/FP59. As a control for the cell sensitivity to the recombinant anthrax toxin, all cell lines were also tested for sensitivity to the furin-activated PrAg/FP59 (Table 1). These results show the wide range and high potency of PrAgU2/FP59. This toxin is capable of targeting a majority of tumor cell lines independently of tumor type and tissue of origin.

**uPAR Expression Levels**

Because sensitivity to PrAgU2/FP59 is expected to depend on the uPAR and uPA content of cells, we evaluated the expression levels of these proteins in the different cell lines, as shown in Table 1.

Table 1. Cell line sensitivity to PrAg/FP59 and PrAgU2/FP59 as well as uPAR expression, total uPA, and total PAI-1 levels

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PrAg/FP59 (IC50; pmol/L)</th>
<th>PrAgU2/FP59 (IC50; pmol/L)</th>
<th>uPAR (receptors per cell)</th>
<th>uPA (ng/mL)</th>
<th>PAI-1 (ng/mL)</th>
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*Cell lines that are not sensitive to the furin-activated PrAg/FP59 and therefore not included in the following tables and analyses.*
determined the status of the uPA/uPAR system in our panel of cell lines (Table 1). Independently of the tumor type, PrAgU2/FP59–sensitive cell lines had significantly higher expression levels of uPAR compared with nonsensitive cell lines (mean uPAR expression = 2,442 and 678 receptors per cell, respectively; \( P = 0.0153 \)). These results indicate that high uPAR expression levels are a contributor to the cytotoxicity of PrAgU2/FP59 and a marker for tumor sensitivity to this toxin. uPAR is, therefore, a possible predictor of tumor sensitivity to PrAgU2/FP59.

**Total uPA and PAI-1 Levels**

We also determined total uPA and PAI-1 levels in the supernatants of our cell line panel (Table 1). PrAgU2/FP59 sensitive cell lines had 10-fold higher mean total uPA levels in their supernatants when compared with nonsensitive cell lines (30.71 and 3.02 ng/mL, respectively; \( P = 0.0058 \)) independently of the tumor type. Mean total PAI-1 levels, on the other hand, were not significantly different in PrAgU2/FP59–sensitive and PrAgU2/FP59–resistant cell lines (399.71 and 79.58 ng/mL, respectively; \( P = 0.08 \); Table 1). These results indicate that, independently of the tumor type, total PAI-1 levels do not contribute to the potency of PrAgU2/FP59 and do not constitute a possible marker for tumor responsiveness to this toxin. High total uPA levels, on the other hand, significantly contributed to the cytotoxicity of PrAgU2/FP59. The presence of uPA is, therefore, a marker of tumor sensitivity to PrAgU2/FP59 and could be used as a predictor for the toxicity of this toxin to tumor cells, independently of the tumor type.

**Comparison of uPA versus uPAR Dependency**

Having disregarded the seven cell lines that were resistant to both PrAg/FP59 and PrAgU2/FP59, we looked at the remaining six cell lines that were only resistant to PrAgU2/FP59. The addition of exogenous sc-uPA totally reversed the resistance of three of these cell lines to PrAgU2/FP59. These cell lines (MDA-MB-435, H1703, and 22rv1) had uPAR expression levels similar to those of sensitive cell lines (1,075, 329 receptors per cell, respectively), but very low levels of uPA expression, explaining why the addition of exogenous sc-uPA increased their sensitivity to PrAgU2/FP59 by 200-fold (IC\(_{50}\) = 54, 3.1, and 31 pmol/L, respectively; Fig. 2). On the other hand, the PrAgU2/FP59 sensitivity of the remaining cell lines (H2030, BxPC3, and LNCaP with 180, 100, and 77 receptors per cell, respectively) was not affected by the addition of exogenous sc-uPA, suggesting that other components of the uPA system were nonfunctional (data not shown). These results show the existence of a threshold of uPAR expression, ~200 receptors per cell, below which cells are resistant to PrAgU2/FP59 independently of uPA levels. uPAR expression is, therefore, an independent predictor of tumor cell sensitivity to PrAgU2/FP59 in addition to total uPA levels.

We also determined the effects of the inhibition of the uPA/uPAR system on the sensitivity of cell lines to PrAgU2/FP59. The H650 non–small cell lung cancer line was 200-fold less sensitive to PrAgU2/FP59 when coincubated with an anti-uPA antibody (IC\(_{50}\) = 1.5 and 254 pmol/L, respectively; Fig. 3). These results were also confirmed on pancreatic cancer, colon cancer, prostate cancer, and breast cancer cell lines (data not shown). These results show that PrAgU2/FP59 is specifically activated by the uPA/uPAR system and, therefore, is selectively toxic to uPA/uPAR–expressing cells.

**ANTXR Expression Levels**

We controlled for the expression of ANTXR in our panel of cell lines by determining the cell sensitivity to the furin-activated PrAg/FP59. Surprisingly, seven cell lines of different tumor origin (non–small cell lung cancer, colon cancer, and breast cancer) were not sensitive to PrAg/FP59 (Table 1). As expected, these cells were also resistant to PrAgU2/FP59. ANTXR expression levels on all these cell lines were <300 receptors per cell and were ~20-fold lower than ANTXR expression levels on cell lines sensitive to PrAg/FP59 but not to PrAgU2/FP59 (Table 2). This shows that ANTXR expression is essential for PrAgU2/FP59.
potency and could be used as a potential first step marker of cell sensitivity to this toxin.

**Normal Cell Sensitivity to PrAgU2/FP59**

To determine the selectivity of PrAgU2/FP59 for tumor cells, we tested its toxicity to five normal human cell types. Cardiomyocytes, cardiac microvascular endothelial cells, renal proximal tubule epithelial cells, and renal cortical epithelial cells were, on average, 300-fold less sensitive to PrAgU2/FP59 than tumor cells (IC₅₀ = 305, 636, and >10,000 pmol/L, respectively), while being as sensitive to the furin-activated anthrax recombinant toxin (Table 3).

Normal human lung fibroblasts were not as resistant to PrAgU2/FP59 as the other normal human cells tested (IC₅₀ = 68 pmol/L). However, the Hill slope of the sigmoidal dose-response curve of all normal cells tested was significantly different of that of tumor cell lines (data not shown), indicating a possible difference in binding and internalization mechanisms between normal and tumor cells. The comparison of the potency of PrAgU2/FP59 to H1299 non–small cell lung cancer cell line (IC₅₀ = 2 pmol/L) and to human cardiac microvascular endothelial cells (IC₅₀ = 636 pmol/L) reveals a 300-fold difference in sensitivity and illustrates the tumor selectivity of PrAgU2/FP59 (Fig. 4). These results confirm the selectivity of PrAgU2/FP59 to uPA/uPAR–expressing tumor cells and indicate the existence of a wide in vitro therapeutic window for the urokinase-activated recombinant anthrax toxin.

**Discussion**

The uPA system is overexpressed on a wide variety of tumor types and, along with matrix metalloproteases, is the most commonly expressed protease system in highly aggressive metastatic tumors. In fact, several studies have shown that patients with high circulating levels of uPA and soluble uPAR have a very poor prognosis (16–18). Multiple attempts have been recently made to inhibit the uPA/uPAR system. These inhibitors, although effective in limiting tumor growth, did not lead to tumor regression, due to the fact that inhibiting the uPA/uPAR system has cytostatic rather than cytotoxic effects on tumor cells (19,20). We and others favor another approach that consists of taking advantage of the expression of the urokinase system on tumor cells to specifically target these cells with fusion toxins (21–23). In this study, we specifically target uPAR-expressing tumor cells with the cytotoxic anthrax recombinant toxin (PrAg/FP59) by modifying the furin cleavage site on PrAg to a uPA cleavage site. PrAgU2/FP59 was highly cytotoxic to a majority of the cell lines present in the large set of tumor cells we tested. This is highly significant because it shows the ability of PrAgU2/FP59 to selectively target urokinase-expressing tumor cells of different tumor types despite binding to both normal and tumor cells through the ubiquitously expressed ANTXRs, tumor endothelial marker-8, and capillary morphogenesis gene-2.

Novel, tumor-selective therapies with well-elucidated molecular mechanisms and easily identifiable markers of tumor sensitivity have allowed the preselection of patients that are most likely to respond. The most recent examples of such therapies are Gleevec, which targets BCR-Abl mutants in chronic myeloid leukemia patients and Herceptin, which targets Her2-neu and is therefore used for the treatment of
breast cancer patients with Her2-neu-overexpressing tumors (24–27).

We looked at four molecular markers that would predict tumor sensitivity to PrAgU2/FP59 and therefore may eventually allow preselection of patients with sensitive tumors. All four of these markers, ANTXR, uPA, uPAR, and PAI-1, are involved in the primary binding, activation, and internalization of PrAgU2/FP59, which constitute the control points for the potency and selectivity of this toxin. The subsequent translocation of FP59 into the cytosol, ADP-ribosylation of EF2, protein synthesis inhibition, and cell death occur independently of tumor cell characteristics. Interestingly, three of four markers proved to be excellent predictors of tumor sensitivity to PrAgU2/FP59. The expression of ANTXR and uPAR as well as the presence of uPA predict tumor cell sensitivity to PrAgU2/FP59, whereas the absence of any one of these markers, especially ANTXR and uPAR, greatly diminishes that sensitivity. Moreover, these markers are very strong predictors because relatively low levels of ANTXR and uPAR expression (>300 and 200 receptors per cell, respectively) as well as total uPA levels (>1 ng/mL) are needed to predict sensitivity of tumor cells to PrAgU2/FP59. Although we did not perform an in-depth analyses of the complex interactions between the four previously mentioned markers, we can conclude the following. ANTXR being the primary binding site of PrAg its presence is essential to the potency of PrAgU2/FP59 independently of uPA and uPAR levels. Also, our data shows that in the absence of uPAR, cell lines are resistant to PrAgU2/FP59 even in the presence of excess amounts of pro-uPA, which indicates that uPAR expression levels are a stronger predictor of the potency of PrAgU2/FP59 than uPA levels.

PrAgU2/FP59 proved to be a highly selective compound because, unlike tumor cell lines, most normal human cells tested were relatively resistant to this toxin. Moreover, the significant difference in the Hill slope of the sigmoidal dose-response curves between normal and tumor cells indicates a possible difference in the kinetics of toxin activation and cell entry (27). This suggests a potentially different mechanism of toxin activation and internalization in normal cells, which might provide the opportunity for novel ways of specific tumor targeting using this family of toxins. Furthermore, the inhibition of the uPA protease system significantly decreased the potency of PrAgU2/FP59 independently of tumor type. This shows the absolute requirement of this toxin for the presence of an active urokinase system at the cell surface for its activation, thus confirming uPA and uPAR as molecular markers and potent predictors of tumor cell sensitivity to PrAgU2/FP59.

The remarkable potency, wide range, and selectivity of PrAgU2/FP59 in vitro indicate the high potential of this approach in the targeting of urokinase-expressing solid tumors. Recent studies have shown the relative safety and selective potency of the systemic administration of PrAgU2/FP59 in a mouse xenograft model (14). However, more extensive investigation is needed to further our understanding of the in vivo safety, potency, range, and selectivity of this toxin.

A growing body of work is increasing our understanding of the selective targeting of tumors with urokinase and matrix metalloprotease—activated fusion toxins. In a recent paper, Liu et al. (12) showed the potency and high tumor specificity of a dual protease complementation system using anthrax toxin activated by both uPA and matrix metalloproteases, which further confirms the potential usefulness of tumor protease-activated toxins and validates this approach for the specific targeting of tumors.

PrAgU2/FP59 is a wide-range, highly selective, and highly potent toxin that specifically targets uPA-expressing tumors, regardless of their tissue origin. This toxin is a very promising potential treatment for all types of urokinase-expressing solid tumors and it deserves further investigation and development.

References


Figure 4. PrAgU2/FP59 cytotoxicity on normal cells versus tumor cells as determined by [3H]thymidine incorporation. PrAgU2/FP59 was ~300-fold more toxic to H1299 non—small cell lung cancer cell line (■) than it was to normal human cardiac microvascular endothelial cells (▲; IC50 = 2 and 636 pmol/L).

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22. Rajagopal V, Kreitman RJ. Recombinant toxins that bind to the urokinase receptor are cytotoxic without requiring binding to the α(2)-macroglobulin receptor. J Biol Chem 2000;275:7566 – 73.


A urokinase-activated recombinant anthrax toxin is selectively cytotoxic to many human tumor cell types


Mol Cancer Ther 2006;5:2556-2562.