Antitumor efficacy of a urokinase activation–dependent anthrax toxin

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Abstract

Previously, we have generated a potent prodruk consisting of modified anthrax toxins that is activated by urokinase plasminogen activator (uPA). The cytotoxicity of the drug, PrAg-U2 + FP59, is dependent on the presence of receptor-associated uPA activity. Local intradermal administration of PrAg-U2 + FP59 adjacent to the tumor nodules in mice with transplanted solid tumors had a potent antitumor effect. In succession of these experiments, we have now investigated the systemic antitumor efficacy of PrAg-U2 + FP59. C57Bl/J mice bearing syngenic tumors derived from B16 melanoma, T241 fibrosarcoma, or Lewis lung carcinoma cells were treated with different mass ratios and doses of PrAg-U2 + FP59. Tumor volumes were recorded daily by caliper measurements. In some experiments, dexamethasone was coadministered. Our data show a significant antitumor effect of systemic administration of PrAg-U2 + FP59 in three syngenic tumor models. Optimal antitumor effect and low toxicity was obtained with a 25:1 mass ratio between the two components (PrAg-U2 and FP59). The experiments show that PrAg-U2 + FP59 displays a clear dose-response relationship with regard to both antitumor efficacy and systemic toxicity. Dose-limiting toxicity seemed to be due to activation of the prodruk by uPA and its receptor in the intestinal mucosa. Concurrent treatment with dexamethasone was found to prevent dose-limiting toxicity. Taken together, these data indicate that uPA-activated toxins may be promising candidates for targeted therapy of human cancers that overexpress uPA and its receptor. [Mol Cancer Ther 2006;5(1):89–96]

Introduction

Urokinase plasminogen activator (uPA) and its cellular receptor (uPAR) are part of a proteolytic system associated with degradation and remodeling of the extracellular matrix in cancer, a hallmark of malignant transformation (1–4). The serine protease uPA is secreted as an inactive proenzyme (pro-uPA) that after binding to uPAR can be converted to active cell surface–associated uPA. uPA in turn activates plasminogen to plasmin. The membrane assembly of the plasminogen activation system components is required to favor and confine plasminogen activation in proximity of the cell surface (5, 6). uPA and uPAR are overexpressed in many types of human cancers, such as ductal breast carcinomas, colon adenocarcinomas, and squamous cell carcinomas in head and neck, lung, skin, and esophagus (2, 7). The fact that both uPA and uPAR are overexpressed in cancer prompted the development of cytotoxic prodrugs that are specifically activated by cell surface–bound uPA (8, 9).

Native anthrax toxin, secreted by the gram-positive bacterium Bacillus anthracis, consists of three individually nontoxic protein components: protective antigen (PrAg), lethal factor, and edema factor. PrAg binds to the specific cellular receptors, tumor endothelial marker 8 (TEM8) or capillary morphogenesis gene 2 (CMG2), and is subsequently cleaved by cell surface–associated furin or furin-like proteases, leading to the dissociation of an amino-terminal fragment (10–14). The carboxyl-terminal part of PrAg remains bound and self-associates to form a heptamer. The heptamer structure enables binding of lethal factor and/or edema factor, which result in insertion of the heptamer in endosomal membranes and subsequent translocation of lethal factor and/or edema factor into the cytosol where they cause cytotoxicity (15–18). The obligatory requirement for proteolytic processing of PrAg at the cell surface provides a way to reengineer the toxin for activation by other cell surface–associated enzymes than furin. Based on the availability of phage display–derived synthetic uPA-cleavable sequences (19), we generated a uPA activation–dependent form of the anthrax toxin protective antigen (PrAg-U2) in which the native furin cleavage site was replaced by a uPA cleavage site (8). To increase the cytotoxicity of lethal factor, a fusion protein [fusion protein 59 (FP59)], consisting of residue 1 to 254 of lethal factor and the catalytic domain of Pseudomonas exotoxin A, was constructed (20). The catalytic domain of Pseudomonas exotoxin A consists of a ADP ribosylation domain that catalyzes the covalent attachment of ADP to elongation factor 2, thereby blocking protein synthesis.
Administration of PrAg-U2 together with FP59 constitutes a potent cytotoxic prodrug requiring activation by uPA. Thus, cells displaying active cell surface–bound uPA and expressing either TEM8 or CMG2 are killed by the drug due to a block of protein synthesis.

uPA and uPAR are either not expressed or only one of the components are expressed at low levels in normal tissues. The expression of both is readily induced during normal tissue remodeling processes, such as wound healing, mammary gland involution, and trophoblast invasion (1, 21, 22). Despite the generally low expression of uPA and uPAR in normal tissues, toxicity in T-cell areas of the spleen and lymph nodes, bone marrow, adrenal cortex, and osteoblasts following administration of PrAg-U2 + FP59 was observed in a previous study (9).

Both in vitro and in vivo, it has been shown previously that activation and cytotoxicity of coadministered PrAg-U2 + FP59 are dependent on the presence of active uPA on the cell surface. In culture, uPAR-negative cells were found to be insensitive to the reengineered toxin and uPA-negative cells were only sensitive when uPA was added to the medium. In addition, cultured cells could be rescued from PrAg-U2 + FP59–induced death by addition of blockers of the interaction between uPA and its receptor (8). Furthermore, PrAg-U2 showed cell-surface uPA-dependent toxin activation in vivo, as revealed by the complete lack of toxic effects in mice deficient in plasminogen, uPA, or uPAR at doses that were lethal to wild-type mice, indicating that the components of the plasminogen activator system are essential for activation of the toxin. In the same study, FP59 or FP59 in combination with a noncleavable form of PrAg was tolerated well by mice even when administered in large doses (200 μg). Moreover, Lewis lung carcinoma tumors grown in plasminogen knockout mice were unresponsive to treatment with the reengineered toxin, confirming that an intact plasminogen activator system is required for the toxin to become activated. Furthermore, local intradermal injections of PrAg-U2 + FP59 beneath transplanted solid tumors showed a significant antitumor effect in three different murine cancers (9).

In succession of these studies, we have now documented the antitumor effect of PrAg-U2 + FP59 after systemic administration and have defined the dose-relationship between the antitumor effects and the toxicity of the prodrug. Moreover, we have found that coadministration of dexamethasone dramatically increases the therapeutic window of the uPA-activated toxin.

Materials and Methods

Tumor Transplantation and Treatment

Lewis lung carcinoma cells and B16 melanoma cells were grown in DMEM with 10% fetal bovine serum. T241 fibrosarcoma cells were grown in DMEM with 1% glutamax/1% nonessential amino acid mix/10% fetal bovine serum. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The cells (10⁶ per mouse) were injected s.c. in the right flank of C57Bl/6j mice. When the tumors reached a volume of ~50 mm³, the mice were allocated in treatment arms of 7 to 13 mice with equal

Table 1. Antitumor effect and toxicity of PrAg-U2 + FP59

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Tumor type</th>
<th>PrAg-U2 (mg/kg)</th>
<th>FP59 (mg/kg)</th>
<th>Ratio</th>
<th>Dexamethasone (5 mg/kg)</th>
<th>Lethality</th>
<th>Kaplan-Meier (P)</th>
<th>No. dead mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (LLC)</td>
<td>0.20</td>
<td>0.068</td>
<td>3:1</td>
<td></td>
<td>8</td>
<td>10 0.0195</td>
<td>0.0117</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.200</td>
<td>3:1</td>
<td></td>
<td>79</td>
<td>10 10</td>
<td>0 0</td>
<td>10</td>
</tr>
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<td></td>
<td>1.20</td>
<td>0.400</td>
<td>3:1</td>
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<td>80</td>
<td>10 0</td>
<td>0 0</td>
<td>10</td>
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<td>1.200</td>
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<td></td>
<td>82</td>
<td>10 0</td>
<td>0 0</td>
<td>10</td>
</tr>
<tr>
<td>2 (LLC); see Fig. 1</td>
<td>0.20</td>
<td>0.068</td>
<td>3:1</td>
<td></td>
<td>0</td>
<td>12 0.0004</td>
<td>NS 0</td>
<td>– 7</td>
</tr>
<tr>
<td>3 (T241); see Fig. 2</td>
<td>0.85</td>
<td>0.034</td>
<td>25:1</td>
<td></td>
<td>0</td>
<td>12 0.0360</td>
<td>NS 0</td>
<td>– 7</td>
</tr>
<tr>
<td>4 (B16)</td>
<td>1.30</td>
<td>0.052</td>
<td>25:1</td>
<td></td>
<td>13</td>
<td>10 0.0002</td>
<td>0.0208</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>0.052</td>
<td>37:5:1</td>
<td></td>
<td>22</td>
<td>10 0.0246</td>
<td>0.0001</td>
<td>4</td>
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<tr>
<td>5 (LLC); see Fig. 3</td>
<td>1.00</td>
<td>0.040</td>
<td>25:1</td>
<td></td>
<td>–</td>
<td>10 0.007</td>
<td>0.003 3</td>
<td>9, 11, and 14</td>
</tr>
<tr>
<td>6 (B16)</td>
<td>1.20</td>
<td>0.048</td>
<td>25:1</td>
<td></td>
<td>11</td>
<td>8 NS</td>
<td>NS 4</td>
<td>5, 5, 8, and 9</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>0.096</td>
<td>12:5:1</td>
<td></td>
<td>11</td>
<td>8 NS</td>
<td>NS 3</td>
<td>6, 7, and 8</td>
</tr>
<tr>
<td>7 (B16); see Fig. 6</td>
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<td>0.048</td>
<td>50:1</td>
<td></td>
<td>20</td>
<td>8 0.0255</td>
<td>0.0083 5</td>
<td>6, 6, 6, 7, and 9</td>
</tr>
</tbody>
</table>

Abbreviations: NS, not significant; LLC, Lewis lung carcinoma.

†As defined in Materials and Methods.

The mice were treated at days 0, 3, 6, 9, 12, and 15 as opposed to days 0, 3, and 6.
median tumor sizes and the treatment was started (day 0). The mice were given i.p. injections of PBS or PrAg-U2 + FP59 in PBS. The construction and purification of both PrAg-U2 and FP59 has previously been described (8, 20). The mice were treated with 3-day intervals, starting at day 0. In one experiment, concurrent daily treatment with 5 mg/kg dexamethasone (VETRANAL, Sigma-Aldrich, Copenhagen, Denmark) was carried out. The experiments were terminated at day 9 with the exception of experiment 5.

**Animals**

Institutional guidelines for animal welfare and experimental conduct were followed in all experiments. All mice (5-8-week-old males) were obtained from Taconic M&B (Ry, Denmark). The mice were conditioned to the new environment for 1 week before starting the experiment. The mice were shaved before tumor cell implantation to facilitate size measurements of the tumors. All mice were ear-tagged to ensure identification of each individual mouse. This was done while the mice were anesthetized by s.c. injections of ketamine (10 mg/kg) and xylazine (1 mg/kg) in isotonic 0.9% NaCl solution. The mice were euthanized by cervical dislocation at the end of each experiment.

**Tumor Growth Analysis**

The sizes of the tumors were determined by daily caliper measurements of two orthogonal diameters in millimeters during the growth phase. The sizes were calculated by the following empirical formula (23):

\[
\text{Tumor size} = d_1 \times d_2^{3/2} \times \pi/6 \times K
\]

\(d_1\) and \(d_2\) are orthogonal diameters in millimeters (adjusted for skin thickness by subtracting 0.5 mm from the measurement) and \(K\) is an empirical constant = 0.67.

Growth-inhibitory effects were evaluated by comparison of tumor sizes (by a two-tailed Student’s t test) and by Kaplan-Meier log-rank analysis. \(P < 0.05\) was considered statistically significant.

**Evaluation of Toxicity**

For each treatment dose, the number of dead mice was recorded and the fractional occurrence of toxic death was expressed by a lethality index calculated by the following formula:

\[
\text{Lethality index} = \frac{\sum (10 - dxt)/n}{10}
\]

10 is the number of days of the experiment, \(dxt\) is the day of event (death of a mouse), and \(n\) is the number of mice in the treatment arm.

The lethality index is the fraction of measurements lost due to toxicity relative to the total number of measurements and, therefore, includes information not only on the number of mice dead in each treatment groups but also on when the mice had died during the experiment. Further, the weight of the mice was recorded on a daily basis.

**Histologic Analysis**

For histologic analysis, intestines, lung, heart, liver, kidney, and spleen were dissected from B16 melanoma-bearing mice treated with 1.96 mg/kg PrAg-U2 + 0.052 mg/kg FP59 or PBS i.p. thrice at 3-day intervals. The tissues were fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin. Five-micrometer sections were deparaffinized, rehydrated, and stained with H&E.

**Cellular Cytotoxicity Assay**

Cells (40,000) were cultured overnight in growth medium in 96-well plates. The cells were treated with PrAg-U2 (0-3,000 ng/mL) combined with FP59 (50 ng/mL) for 24 hours. Cell viability was assayed by adding 40 μL of 5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich) at 48 hours. The cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide for 1 hour, the medium was removed, and the purple pigment produced by the cells was dissolved in 200 μL DMSO. Absorbance was measured at \(A_{570}\). Cell viability (percentage of control) was plotted versus \(\log_{10} \text{PrAg-U2 concentration}\).

**Results**

**Significant Antitumor Effect of PrAg-U2 + FP59 Administered Systemically**

We first assayed the antitumor effect of the uPA-activated toxin after i.p. administration to Lewis lung carcinoma–transplanted mice. Mice bearing solid s.c.
tumors of \( \approx 50 \text{ mm}^3 \) volumes were allocated to treatment arms with different doses of PrAg-U2 + FP59 thrice with 3-day intervals (days 0, 3, and 6). These mice were treated with different absolute doses, but all with a mass ratio of PrAg-U2 to FP59 of 3:1, in accordance with the ratio previously applied as local, intradermal/peritumoral injections (9). The lowest dose (0.2 mg/kg PrAg-U2 + 0.068 mg/kg) resulted in significant antitumor effect (\( P < 0.05 \)), whereas the higher doses were lethal (Table 1, experiment 1).

**Comparison of Different Ratios of PrAg-U2/FP59**

Anthrax receptors are ubiquitously expressed in virtually all types of mammalian cells, which implies that a relatively large amount of PrAg-U2 protein is necessary to saturate these binding sites. The efficiency of this toxin system may, therefore, be dependent on the ratio between the two components, PrAg-U2 and FP59. To investigate this and approximate the optimal ratio between the two components, we compared the antitumor effect of three different doses of the toxin with PrAg-U2 + FP59 treatment. The sizes of the tumors are expressed as mean tumor size in mm\(^3\) ± SEM. The symbols correspond to the symbols used in top.

To investigate if prolonged treatment of the mice could further inhibit the tumor growth, we decided to extend the treatment period. Lewis lung carcinoma–bearing mice were treated with 1.00 mg/kg PrAg-U2 + 0.04 mg/kg FP59 at days 0, 3, 6, 9, 12, and 15. The treatment resulted in a significant antitumor effect; however, the extended treatment did not suppress tumor growth completely, indicating that not all uPAR-positive tumor cells were killed by the treatment (Fig. 3; Table 1, experiment 5).

Taken together, these results show that PrAg-U2 + FP59 is active when administered systemically and that the prodrug induces a significant growth delay in Lewis lung carcinoma, T241, and B16 transplanted tumors.

**Dose-Limiting Toxicity after Systemic Treatment**

In the experiments described above, we observed a dose-limiting toxicity with the highest doses. As a measure of this toxicity, we calculated a lethality index, which reflects not only how many of the mice that had died but also how early they had died (see Materials and Methods). To investigate if increasing the dose of either of the different doses of PrAg-U2 + FP59 were applied, two doses with a ratio of 25:1 of PrAg-U2 to FP59 (0.85 mg/kg PrAg-U2 + 0.034 mg/kg FP59 and 1.70 mg/kg PrAg-U2 + 0.068 mg/kg) and one dose of PrAg-U2 + FP59 with a ratio of PrAg-U2 to FP59 of 50:1 (2.00 + 0.040 mg/kg). The highest dose with a mass ratio 25:1 and the dose with a ratio of 50:1 both resulted in significant tumor growth inhibition (Fig. 2; Table 1, experiment 3). In the experiment with B16-bearing mice, three ratios of PrAg-U2 + FP59 were applied. FP59 was held at a constant dose (0.052 mg/kg) and the doses of PrAg-U2 were increased to give ratios of 25:1, 37.5:1, and 50:1. All these doses resulted in significant tumor growth inhibition of the B16 melanomas (Table 1, experiment 4).
two components led to an increase in lethality in B16-bearing mice, we first kept the dose of FP59 at a constant level of 0.052 mg/kg, whereas the dose of PrAg-U2 was varied from 1.30, 1.96, or 2.61 mg/kg (Table 1, experiment 4). The doses resulted in lethality indexes of 13%, 22%, and 36%, respectively, demonstrating that increasing the dose of PrAg-U2, when FP59 is kept at a constant level, results in an increase in lethality. Analogous to what is observed by increasing PrAg-U2, the lethality was increased from 11% to 50% in B16-bearing mice, when the dose of FP59 was increased from 0.048 to 0.144 mg/kg and PrAg-U2 was held constant at 1.20 mg/kg (Table 1, experiment 6).

All together, seven different ratios of PrAg-U2 to FP59 were tested in the experiments, ranging from 3:1 to 50:1. To analyze the relationship between doses, ratios of the two components, and lethality, we plotted PrAg-U2 doses against the doses of FP59 for each ratio (Fig. 4). For all the ratios tested, this plot shows that an increase in dose results in an increase of the lethality index.

Gross inspection of mice treated with the highest doses of PrAg-U2 + FP59 revealed ulcerations of the anal region and edema of the small intestines (Fig. 5B). No other gross pathology was noted in any mice as a result of the treatment. Histologic investigation of intestines of mice treated with either vehicle or 1.96 mg/kg PrAg-U2 + 0.052 mg/kg FP59 revealed that the intestines of the toxin-treated mice were clearly affected by the treatment. The villi in the small intestine were severely inflamed, the tips of the villi were necrotic, and occasionally necrotic tissue was shed into the intestinal lumen (Fig. 5D). The villi of the vehicle-treated animals appeared normal. No histologic signs of toxicity were observed in the liver, lung, kidneys, heart, and spleen of the toxin-treated B16 melanoma-bearing mice (data not shown).

Dose-Limiting Toxicity Is Efficiently Inhibited by Dexamethasone

To investigate if supplying the mice with an anti-inflammatory drug could attenuate the dose-limiting toxicity, daily treatment with the glucocorticoid dexamethasone was initiated. B16-bearing mice were treated with...
2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59, resulting in a lethality index of 20%. Concurrent treatment with dexamethasone reduced the lethality index to 1% (Fig. 6; Table 1, experiment 7). In the same experiment, we recorded the weight of the mice on a daily basis. Mice treated with 2.40 mg/kg PrAg-U2 + 0.048 mg/kg had a significant weight loss compared with PBS-treated mice ($P < 0.05$). Interestingly, when the mice received dexamethasone in addition to PrAg-U2 + FP59, no weight loss was observed (Fig. 7). Because dexamethasone has previously been reported to have growth-inhibitory effects in B16 melanoma tumors (24), we specifically addressed this potential bias. Daily dexamethasone treatment of 5 mg/kg for 9 days did not induce significant growth delay of B16 melanomas.

Direct Killing of Tumor Cells by PrAg-U2 + FP59

To clarify whether the antitumor effect of PrAg-U2 + FP59 observed in vivo is caused by direct killing of the tumor cells or by affecting the stroma, we analyzed the cytotoxic effect of the toxin in vitro by a colorimetric cytotoxicity assay. All three murine cell lines were sensitive to PrAg-U2 + FP59 treatment (data not shown) in a dose-dependent manner, demonstrating that the cells express the component required for activation and intoxication of the cells. Furthermore, the results indicate that the in vivo antitumor effect is mediated at least in part by a direct effect on the tumor cells.

**Discussion**

The well-documented overexpression of uPA and uPAR in several types of cancer makes the plasminogen activation system an attractive candidate for targeted therapy. In many experimental in vivo studies, the aim has been to block the plasminogen activation system either with inhibitors of the active site of uPA (25–27) or with inhibitors of the uPA/uPAR interaction (for review, see ref. 7). In the present approach, we have used a two-component prodrug system, PrAg-U2 + FP59, which consists of modified anthrax toxins that specifically kill cells dependent on the presence of cell surface–associated uPA activity, resulting in a direct cytotoxic effect both in vitro and in vivo (8, 9).

In our previous in vivo studies, local administration of the prodrug resulted in a substantial antitumor effect and, in some cases, tumor eradication. PrAg-U2 + FP59 was injected intradermally in the area surrounding the tumor nodules to obtain high local concentrations of drug and around the tumors (9). Although local administration of PrAg-U2 + FP59 in clinical settings may be feasible and even desirable in special types of malignancies, such as head and neck cancer or brain cancer, most cancer treatment is systemic. We have, therefore, investigated the antitumor activity of PrAg-U2 + FP59 after systemic administration. Importantly, we find that the modified toxins have significant antitumor effects after i.p. administration. The optimal ratio between PrAg-U2 and FP59 for systemic use is 25:1 instead of the 3:1 ratio used for local administration. The reason for the need of a larger excess of PrAg-U2 to FP59 for systemic administration...
is probably the existence of a “sink effect” due to the ubiquitous expression of PrAg receptors in the normal tissues, particularly CMG2 and, to some extent, TEM8 (10, 28). The antitumor effect of systemically administered drug was clearly dose dependent. However, high doses of PrAg-U2 + FP59 also elicited toxicity. By dose optimization, we could approximate the optimal treatment dose that shows a significant growth inhibitory effect and at the same time low toxicity. However, a definitive determination of the therapeutic window of the prodrug is complicated by the two-component nature of the drug and, thus, could not be defined by an absolute or single value because it depends on the dose of each of the two components. If a low dose of FP59 is administered, a high dose of PrAg-U2 can be administered without toxicity and vice versa.

PrAg-U2 + FP59 targets cells that harbors receptor-bound uPA on their surface. In some types of cancer, uPA and uPAR are often expressed by the stromal cells and only in a subpopulation of the cancer cells, which means that the antitumor effect of PrAg-U2 + FP59 in such cases may be obtained either by direct killing of cancer cells or indirectly by destruction of the cancer-associated stroma. In colon and breast cancer, uPAR is predominantly expressed by macrophages, whereas uPA is produced and secreted mainly from myofibroblasts (29–33). However, uPAR is also expressed by budding cancer cells in colon cancer (34) and may also be expressed at a certain level in some breast cancer cells (30, 35). This expression pattern is clearly distinct from that observed in squamous cell carcinomas, in which the predominant expression of uPA and uPAR is seen in the cancer cells as typified by squamous cell cancer of the skin (36) and esophagus (37). Lung, head and neck, cervical, and esophageal squamous cell carcinomas may thus be good candidates for PrAg-U2 + FP59–based therapy aiming at targeting the cancer cells directly.

The systemic toxicity of uPA-activated toxin may be related to an inflammatory response elicited by PrAg-U2 + FP59. We therefore anticipated that steroid treatment with dexamethasone would alleviate toxicity. In addition to the anti-inflammatory effect, dexamethasone has been reported to decrease the level of uPAR expression (38). Co-administration of dexamethasone with a high dose of PrAg-U2 + FP59 completely eliminated toxicity compared with the group that received PrAg-U2 + FP59 alone. Whether dexamethasone abrogates a general systemic inflammatory condition or a local tissue-specific damage in the PrAg-U2 + FP59–treated mice needs to be investigated in more detail.

We have observed both gross and histologic signs of intestinal affection in PrAg-U2 + FP59–treated mice that were clearly suffering from malaise. The finding of strong inflammation and necrosis in the tip of the intestinal villi indicate that this part of the villi is especially vulnerable to the toxin. The reason for this may be that both uPA and uPAR are expressed in the villi in the normal intestine. uPAR is expressed in the epithelial mucosa cells lining the villi and uPA is expressed in fibroblast-like cells located just beneath the epithelium and may hence be found at the distal epithelium (39–41). In addition, CMG2 and TEM8 is expressed in the small intestine (10, 42, 43). The colocalized expression of the components required for activation of the prodrug may thus explain the specific damage to the epithelial cells at the tip of the villi. In support of this hypothesis, our previous studies showed that plasminogen activator inhibitor-1–deficient mice presented edema and hemorrhage of the small intestine even at very low PrAg-U2 + FP59 doses (9).

The mechanism of in vivo antitumor effect of PrAg-U2 + FP59 is still not clearly understood and need further analysis. Evidence from our previous studies and studies by others (9, 44) points both at a direct cytotoxic effect of PrAg-U2 + FP59 toward tumor cells and also at an antiangiogenic effect. We observed pronounced tumor vascular damage with angiectasis, vascular stasis, and hemorrhage in PrAg-U2 + FP59–treated Lewis lung carcinomas (9). However, the endothelial damage might be secondary to tumor cell cytotoxicity because the antitumor effect of PrAg-U2 + FP59 in Lewis lung carcinoma–transplanted uPAR−/− mice was equal to the effect seen in wild-type mice (9). Histologic investigations of tumor tissue from PrAg-U2 + FP59–treated Lewis lung carcinoma–bearing mice, previously done, showed cytoplasmic shrinkage, nuclear condensation, and cessation of BrdUrd incorporation into the tumor cells. In addition, TUNEL staining revealed no increase in tumor cell apoptosis, suggesting that the tumor cells were undergoing necrotic cell death and not apoptosis. Furthermore, we have now shown that PrAg-U2 + FP59 is capable of killing Lewis lung carcinoma, B16 melanoma, and T241 fibrosarcoma cells in vitro by cytotoxicity experiments. It has previously been shown that Lewis lung carcinomas express both uPA (45) and uPAR (46). Taken together, these data strongly suggest that the antitumor effect is mediated, at least in part, by direct killing of the tumor cells.

In conclusion, we here provide evidence of potent antitumor effect after systemic administration of an uPA-activated toxin and, furthermore, the possibility to block the dose-limiting toxicity by coadministration of dexamethasone. These findings warrant further development of uPA-activated toxins in the clinic for specific targeting of cancers with overexpression of uPA and uPAR.

References


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