Intratumoral Generation of 5-Fluorouracil Mediated by an Antibody-Cytosine Deaminase Conjugate in Combination with 5-Fluorocytosine

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ABSTRACT

Cytosine deaminase (CD) is a microbial enzyme that can convert the antifungal agent 5-fluorocytosine (5-FC) into the antitumor agent, 5-fluorouracil (5-FU). The enzyme was chemically conjugated to the L6 monoclonal antibody, forming a conjugate that bound to antigens on the H2981 lung adenocarcinoma. Detailed studies were undertaken to determine the extent to which L6-CD generated 5-FU in tumor-bearing mice. Very high tumor:blood ratios of L6-CD (42:1) in vivo were obtained by injecting the conjugate followed 24 h later by an antidiotopic antibody that could bind to circulating L6-CD but not to L6-CD that was bound to H2981 cells. As a result, significantly more 5-FC could be administered (>800 mg/kg) than 5-FU (90 mg/kg). L6-CD converted 5-FC into 5-FU such that the L6-CD/antidiotopic monoclonal antibody/5-FU combination resulted in 17 times more intratumoral 5-FU compared to systemic 5-FU administration. The conversion was antigen dependent since much lower intratumoral 5-FU levels were obtained in H3719 tumors that failed to localize L6-CD. The conversion of 5-FC into 5-FU was low in blood, kidneys, and liver. This demonstrates that a major increase in intratumoral drug concentrations can be attained with an monoclonal antibody-enzyme conjugate in combination with an anticancer prodrug compared to systemic drug therapy.

INTRODUCTION

Colorectal carcinoma, especially when disseminated to distal sites, has proven to be refractory to many chemotherapeutic strategies. Approximately one-half of the patients diagnosed with the disease ultimately develop unresectable lesions resulting in 80,000 lives being lost annually in the United States alone (1). The use of bolus 5-FU3 has been standard chemotherapy for the past two decades, despite a reported response rate of only 5–25% (1, 2). Some progress has been made in clinical trials by combining 5-FU with a variety of biochemical modulators such as levamisole (3), leucovorin (4), and a-interferon (5). However, in these studies the main effects have been on response rates rather than on survival.

The effectiveness of 5-FU is limited by the systemic toxicity associated with treatment. Higher doses or increased dose intensity of 5-FU may prove beneficial in improving prognosis since treatment of gastrointestinal neoplasm with 5-FU exhibits a clear dose response, although, unfortunately, also a dose-toxicity relationship (6). Attempts to combine 5-FU with other drugs that produce a response in colorectal carcinoma (e.g., nitrosoureas and mitomycin C) have been hampered by the necessity to reduce the doses compared to single modality treatment in order to prevent toxicity (7). Furthermore, there is persuasive evidence that aggressive drug regimens could improve clinical outcomes by limiting the development of acquired drug resistance (8).

MATERIALS AND METHODS

Materials

HPLC grade methanol, ethyl acetate, and 2-propanol were purchased from Fisher. Reagent grade formic acid, phosphoric acid, glacial acetic acid, and ammonium hydroxide were obtained from Mallinckrodt, and 5-FC and 5-FU were obtained from Sigma Chemical Co. The human lung adenocarcinoma cell line H2981 (12) and human colon carcinoma line H3719 were established at Bristol-Myers Squibb (Seattle, WA). [3H]5-FU and [3H]5-FC were purchased from Moravek Biochemicals (Brea, CA). The hybridoma producing the antiidiotype antibody 13B recognizing the L6 antibody was grown as ascites and purified by protein A Sepharose chromatography (13). The L6-CD conjugate was prepared using two previously described methods producing a thioether linkage between the two proteins (11, 14). Both conjugation methods yielded materials of M, 200,000 that behaved indistinguishably in all experiments in which they were compared.

In Vivo Studies. Female nude mice (aged 8–10 weeks; Harlan-Sprague-Dawley, Indianapolis, IN) were inoculated with tumor cells from in vitro sourcing, and the tumors were maintained by in vivo passaging using sections of approximately 32 mm3 implanted s.c. into the right hind flank. Tumors of passage 3 through 8 were used in the experiments described. L6-CD in PBS (pH 7.2) was injected via the tail vein at the doses described. 5-FU, 5-FC, and the 13B antidiotopic antibody were injected i.p. Mice were anesthetized with halothane before bleeding via the retroorbital sinus. Plasma samples and tissue samples were frozen in liquid nitrogen immediately after collection. Using plasma and tumors spiked with known amounts of CD, it was found that the enzyme activity was not significantly altered by the freezing procedure. MTDs were determined by injecting groups of six tumor-bearing mice with either drug, prodrug, or the prodrug conjugate with three weekly rounds of treatment. The animal weight and survival were noted.

Radioactive Localization. The L6-CD conjugate was labeled with 125I using Iodogen (Pierce Chemical Co.) to a specific activity of 1010 cpm/mg with no loss of immunoreactivity. The conjugate (300 µg/25 g mouse) was injected via the lateral tail vein (three mice/group). At various time points, the mice were anesthetized and bled via the retroorbital sinus and then sacrificed before removal of various organs. The tissue samples were weighed and counted on a Beckman Gamma 550 counter. The percentage of the injected dose was calculated assuming a 22-g mouse to have a blood volume of 2.18 ml.

Conjugate Clearance Studies. CD activity was used to assess conjugate clearance from mice (3–4 mice/group) that were treated (i.v.) with L6-CD (300 µg). To measure CD activity in blood, samples were taken from the orbital...
plexus of anesthetized mice into a heparinized tube and centrifuged; the plasma was frozen at ~70°C until use. After thawing, 50-µl plasma samples were added to 950 µl of 5-FC (3 mM in PBS) and incubated at 37°C. At various times, 50-µl aliquots were removed and quenched with 1.0 ml of 1 M hydrochloric acid; the concentrations of 5-FC and 5-Ri were determined spectrophotometrically (1) using:

\[
\begin{align*}
5-FC [\text{mM}] &= 0.119 (A_{290}) - 0.025 (A_{255}) \\
5-FU [\text{mM}] &= 0.185 (A_{293}) - 0.049 (A_{290})
\end{align*}
\]

One unit of activity represents 1 µmol 5-FU formed/min at 37°C.

Enzyme levels in tumors were measured by homogenizing the tumor (~200 mg) in 450 µl of PBS containing 1 µg/ml aprotinin, 30 µg/ml phenylmethylsulfonyl fluoride, and 5 mM EDTA. To this was added 50 µl of 10 nM [3H]5-FC (1.3 Ci/mole). At various intervals, 50-µl aliquots of each reaction were removed and quenched in 0.5 ml methanol. The samples were evaporated to dryness in a Speed Vac Concentrator (Savant, Hicksville, NY). The residue was suspended in 20 µl of methanol and centrifuged; and 5 µl of supernatant was applied to Baker-Flex IB-F TLC plates (J. T. Baker, Phillipsburg, NJ) which were developed in 6:1 chloroform:methanol (RF: 5-FC = 0.2, 5-Ri = 0.7). Sections of the plates containing the UV absorbing spots corresponding to the positions of 5-FC and 5-Ri were cut out and placed into scintillation vials. These sections were counted in a Beckman 8600 scintillation counter with Optifluor (Packard Instruments, Downers Grove, IL). The relative percentages of 5-FC and 5-FU and the amount of enzyme was determined by comparison to standards. Blood levels of cytotoxic deaminase activity were determined as for the tumor by adding 50 µl of plasma to 400 µl of PBS prior to the addition of [3H]5-FC.

**HPLC Conditions**

5-FC and 5-FU were separated isocratically on a Hewlett Packard HP1090 M Liquid Chromatograph with UV detection at 280 nm. A Brownlee RP-18 (5 µm packing; 2.1 x 220 mm) column and a matching 30-mm guard cartridge (Applied Biosystems Inc., San Jose, CA) were used with a flow rate of 0.2 ml/min. The mobile phase was 10 mM formic acid and 50 mM phosphoric acid adjusted to pH 3.5 with ammonium hydroxide. 5-FC and 5-FU eluted in 4.6 and 6.6 min, respectively. It was necessary to allow at least 1 h between tumor samples and 15 min between plasma samples to ensure that the column was clean before being used again. During analyses, samples were maintained at 5°C in a temperature-controlled sample chamber.

**RESULTS**

**Toxicity and Clearance of 5-FC and 5-FU**

The suitability of 5-FC as a prodrug for activation by a mAb-CD conjugate in vivo was determined. 5-FU in nude mice was nontoxic at all doses that could logistically be given and could be administered at 2.4 g/kg (a dose limited by solubility) in three weekly injections. In contrast, the MTD of 5-FU in these experiments was 90 mg/kg using the same schedule. The difference in toxicities was not due to accelerated clearance since both compounds had similar half-lives (5-FC, \( t_{1/2} = 12 \) min; 5-FU, \( t_{1/2} = 16 \) min). The fact that there was no apparent conversion of 5-FC to 5-FU as late as 64 min after 5-FC injection (data not shown) confirms the lack of CD activity in non-conjugate treated mice. However, when the L6-CD immunono conjugate (300 µg) was administered 72 h prior to the initiation of produrg, the MTD of 5-FC was only 120 mg/kg.

**Clearance of the Antibody-Enzyme Conjugate**

In order to reduce the toxicity of 5-FC following L6-CD conjugate administration, we investigated the ability of the L6 antiidiotopic antibody 13B (13) to accelerate the clearance L6-CD. The conjugate (300 µg) was administered, followed 24 h later by injection of 13B (200 µg) i.p. The 24-h time point for administration of the antiidiotype antibody was chosen to allow time for antibody accumulation at the tumor. 13B was administered i.p. since previous studies using i.v. administration caused some mortality. The slower entry to the circulation via i.p. administration was probably beneficial. In 13B-treated animals, only 0.3 ± 0.2% of the CD enzymatic activity remained in the blood 48 h post conjugate treatment (Fig. 1). In animals that did not receive the 13B mAb, the blood contained 41-fold more CD activity (12.2 ± 0.5% of the injected dose). At 48 h after the injection of the antiidiotype (72 h post conjugate), the circulating enzyme activity was further reduced to 0.12 ± 0.07% of the injected dose compared to 8.2 ± 0.3% in mice that were not treated with 13B. This represents a 68-fold decrease in blood-borne CD activity.

Table 1 presents the results of experiments to evaluate the effects of the antiidiotype antibody on intratumoral CD levels. The mice used in these experiments were previously implanted (s.c. in the flank) with either the H2981 tumor (binds L6) or the H3719 tumor (does not bind L6) xenografts. L6-CD (300 µg) was administered i.v. followed 24 h...
later by 200 μg of 13B, which was administered i.p. Tumor and plasma samples were collected immediately prior to and 24 and 48 h after 13B administration. CD activity was then measured using the conversion of [3H]5-FC to [3H]5-FU in both plasma and tumors. It was found that the H2981 and H3719 tumor:blood CD activity ratios were constant at 24 and 48 h in animals that did not receive 13B administration. The results are ±SE 30%.

Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Time postconjugate administration (h)</th>
<th>% injected dose CD activity/g tissue</th>
<th>L6-CD</th>
<th>L6-CD + 13B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor Blood Ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2981</td>
<td>24</td>
<td>6.3/6.6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3.9/2.9</td>
<td>1.3</td>
<td>2.9/0.16/18</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.1/1.7</td>
<td>1.8</td>
<td>2.5/0.06/42</td>
</tr>
<tr>
<td>H3719</td>
<td>24</td>
<td>7.7/6.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3.6/4.5</td>
<td>0.8</td>
<td>0.18/0.22/0.8</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2.9/2.7</td>
<td>1.1</td>
<td>0.07/0.09/0.8</td>
</tr>
</tbody>
</table>

* Mice were treated (i.v.) with L6-CD (300 μg) at the indicated times, tumor and blood were removed, and CD activities were determined. The results are ±SE 30%.

HPLC Analysis of 5-FC and 5-FU

A reversed phase chromatography method was developed to simultaneously analyze 5-FC and 5-FU in both plasma and tumor samples. Under the conditions established, baseline separation of 5-FC and 5-FU was obtained with complete resolution from all other plasma or tumor-derived peaks (Fig. 3). Extraction efficiencies obtained from spiked plasma were 50% for 5-FC and 78% for 5-FU. Control experiments demonstrated that CD was inactivated by the organic solvents used to quench enzyme activity. Extraction efficiencies obtained from spiked tumors were 61% for 5-FC and 66% for 5-FU. Although most of the in vivo work was in the range of 10–3000 nmol drug/ml in blood and 100–2500 nmol drug/g in tumors, the drugs could be detected at levels less than 4 nmol/ml in blood and 6 nmol/g in tumors.

Tumor and Blood Levels of 5-FU from Targeted and Systemic Therapies

The blood and tumor levels of 5-FU were measured in the H2981 carcinoma xenografts in nude mice that received the L6-CD/13B/5-FC combination and were compared to 5-FU levels resulting from systemic 5-FU treatment. The blood concentration of 5-FU reached a maximum of 28 μg/ml 10 min after injection of 5-FU (90 mg/kg) and then declined with a half-life of approximately 12 min (Fig. 4A). This was comparable to the enzyme/prodrug combination in which 300 μg of L6-CD was administered, followed 24 and 48 h later by 13B (200 μg) and 5-FC (800 mg/kg), respectively. In these animals, the blood level of 5-FU was 33 μg/ml at 15 min and then declined to less than 5 μg/ml at 45 min.

The 5-FU concentrations in the tumors of the same animals were also measured (Fig. 4B). When systemic 5-FU was given, the 5-FU level reached a maximum of 50 μg/gm of tumor 15 min post 5-FU injection. The intratumoral 5-FU level in animals receiving the L6-CD/13B/5-FC regimen was much higher. At 10 min post 5-FU injection, the 5-FU level was 185 μg/g and continued to climb until it reached a maximum of 365 μg/g at 45 min. The 5-FU level resulting from the enzyme/prodrug combination was above the maximal level attained with systemically administered 5-FU for 90 min after the administration of 5-FC. The peak concentration of 5-FU resulting from the targeted therapy was 7 times greater than that resulting from systemic 5-FU treatment, and there was a 17-fold increase in the area under the curve of intratumoral 5-FU from the targeted therapy compared to systemic drug administration.
with 5-Ri (90 mg/kg) (0) or L6-CD (300 µg; t = 0 h), 13B mAb (200 µg; t = 24 h), and 5-FC (800 mg/kg; t = 48 h) (●). Animals were sacrificed at the indicated times, and tumor and blood sample analyses were done for 5-Ri levels by HPLC.

were similar to those found with the H2981 tumors (Fig. 4B). In the binding the L6-CD conjugate. Thus, higher drug concentrations can be attained in tumors capable of 5-PU was given systemically, the intratumoral 5-PU concentrations repeated using the nonbinding control tumor H3719 (Fig. 5). When 5-FU was given systemically, the intratumoral 5-FU concentrations were similar to those found with the H2981 tumors (Fig. 4B). In the animals receiving the targeted regimen, the maximum concentration of 5-FU (50 µg/g at 60 min) was 7-fold less than the binding tumor. Thus, higher drug concentrations can be attained in tumors capable of binding the L6-CD conjugate.

Organ Accumulation of 5-FC and 5-FU

The levels of 5-FU and 5-FC were measured in the livers and kidneys of mice receiving the targeted therapy since these organs are involved in mAb conjugate and drug clearance. Table 2 compares 5-FU and 5-FC levels in these organs with the tumor and blood levels at 30 min post 5-FC administration. The amount of 5-FU in both the liver (12 µg/kg) and kidney (18 µg/kg) were similar to that measured in the blood (15 µg/kg). However, the ratio of 5-FC:5-FU was 13 in the liver, 23 in the kidney, and 29 in the blood, indicating very little prodrug conversion in these tissues. This is in stark contrast to the tumor in which the drug was the major component with 3.3 times as much 5-FU as 5-FC. Thus, the 5-FU:5-FC ratio was ~100-fold greater in the tumor than the blood and 75- and 44-fold greater than in the kidneys and liver, respectively. This data, therefore, supports the notion than the tumor is the major site for prodrug activation.

DISCUSSION

Localized in vivo generation of the anticancer agent 5-FU from 5-FC by encapsulated CD has been described previously in studies in which a permeable membrane that contained immobilized CD was surgically implanted near a tumor site prior to systemic administration of 5-FC (15). Also pertinent to this study is a recent report concerning the selective generation of 5-FU from 5-FC by genetically modified colorectal carcinoma cells expressing CD activity (16). Although significant antitumor activities were obtained in both cases, the question remains whether these approaches are applicable for the treatment of disseminated tumors and/or tumors not amenable to surgical intervention. The use of mAb conjugates for the generation of 5-FU should not be subject to such restrictions since mAbs have been shown to localize in surgically inaccessible tumors following administration (17).

5-FC is, in some respects, an ideal prodrug for enzyme activation due to its almost complete lack of toxicity as well as the fact that both it and 5-FU are clinically approved with well-studied in vivo properties. In the nude mouse, the maximum amount of 5-FC that could be administered was limited only by its solubility and the volume that could safely be injected. This is consistent with previous observations in several species, including man, that 5-FC is not converted to 5-FU due to the lack of an endogenous enzyme activity that can catalyze this reaction (18, 19).

In order to maximize the effectiveness of 5-FC in combination with mAb-CD conjugates, it was necessary to minimize the adventitious conversion of 5-FC to 5-FU by blood-borne L6-CD conjugate. Conceivably, monovalent mAb fragments could be used for conjugation that would clear more rapidly and increase the tumor:blood ratio compared to whole mAb conjugates. However, the reduction in valency of L6 has been shown to reduce its binding to L6 antigens (20).

It would be expected that IA fragments conjugated to CD would have an advantage due to the lack of an endogenous enzyme activity that can catalyze this reaction (18, 19).

To demonstrate immunological specificity, this experiment was repeated using the nonbinding control tumor H3719 (Fig. 5). When 5-FU was given systemically, the intratumoral 5-FU concentrations were similar to those found with the H2981 tumors (Fig. 4B). In the animals receiving the targeted regimen, the maximum concentration of 5-FU (50 µg/g at 60 min) was 7-fold less than the binding tumor. Thus, higher drug concentrations can be attained in tumors capable of binding the L6-CD conjugate.

Table 2 Tissue levels of 5-FC and 5-FUa Conversion relative to 5-FC

<table>
<thead>
<tr>
<th>Tissue</th>
<th>5-FC (µg/g)</th>
<th>5-FU (µg/g)</th>
<th>Conversion relative to blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>18</td>
<td>324</td>
<td>23:1</td>
</tr>
<tr>
<td>Liver</td>
<td>12</td>
<td>152</td>
<td>13:1</td>
</tr>
<tr>
<td>Blood</td>
<td>15</td>
<td>435</td>
<td>29:1</td>
</tr>
<tr>
<td>Tumor</td>
<td>307</td>
<td>90</td>
<td>0.3:1</td>
</tr>
</tbody>
</table>

a Mice were treated with L6-CD (300 µg, i.v.) followed at 24 h by 13B (200 µg, i.p.). At 48 h, mice were injected with 5-FC (800 mg/kg), and 30 min later, tissues were removed. HPLC analysis of homogenized tissues was used to quantify organ levels of 5-FC and 5-FU.
binds to tumor antigens. An alternative approach has been reported using a clearing mAb raised to the enzyme portion of a mAb carboxypeptidase conjugate (24). Since the anticarboxypeptidase mAb was capable of inhibiting enzyme activity bound at the tumor site, it was necessary to accelerate its removal from the circulation by chemical glycosylation. As a result, the ability of this mAb to lower unbound enzyme activity in blood and peripheral tissues would most likely be limited. In contrast, the antiidiotype antibody 13B does not need to be confined to the vascular compartment, a factor we believe to be important in maximizing the tumor:blood mAb:CD ratio. By accelerating the clearance of L6-CD with 13B, it was possible to administer in excess of 800 mg/kg of 5-FC with no toxicity, compared to only 90 mg/kg of 5-FC alone. A complementary approach to the use of antiidiotype mAbs for mAb-CD conjugate clearance has recently been described in which a mAb against CD that does not affect CD activity was used to clear circulating conjugate (14).

Using 13B in the clearance protocol, we compared tumor and blood levels of 5-FC from systemic and targeted therapy. The blood levels of 5-FC with the two regimens were similar, consistent with the fact that both treatments were performed at or near the MTDs. However, in stark contrast, the peak tumor 5-FC level in the targeting strategy was 7-fold higher, and the area under the curve was 17-fold greater (21—23). Studies with model predictions also indicated that the major route of excretion of small molecular weight 5-FC was renal. The major route of elimination of 5-fluorouracil (5-FU) is the liver, whereas that of 5-FC is the kidneys, there would be the potential for generating high levels of tumor:blood ratio (~2), which seems most likely that the 5-FC generated by the L6-CD was generated from intratumoral 5-FC. Importantly, the 5-FC at the tumor did not return rapidly to the systemic circulation. At 10 min, the 5-FC tumor:blood ratio was 3, and at 60 min, the ratio increased to 150.

One of the nuances of mAb-enzyme conjugates for prodrug activation is that the major route of excretion of small molecular weight drugs is through the kidneys (18, 19), while high molecular weight conjugates are cleaved through the liver. The prodrug and enzyme should therefore be spatially separated during clearance, reducing adventitious enzymatic prodrug conversion. If the antiidiotype mAb and L6-CD were to form immune complexes that accumulated in the kidneys, there would be the potential for generating high levels of kidney-associated 5-FC. This was not found to be the case.

We have shown that the L6-CD/13B/5-FC combination can be used to deliver substantially higher levels of 5-FC to an experimental tumor than can be achieved via systemic administration. The use of such an approach clinically may result in increased efficacy compared to 5-FC alone or combinations of 5-FC with other agents (e.g., leucovorin, levamisole, etc.). Additionally, since a given amount of 5-FC can be delivered with much less systemic toxicity using this targeting strategy, it may be possible to increase the doses of other drugs used in combination chemotherapy. This might significantly improve chemotherapeutic efficacy.

**ACKNOWLEDGMENTS**

We acknowledge the contribution of Dr. K. E. Hellström in suggesting the use of the 13B antiidiotype antibody. We also thank Deb Mahan and Mark Stebbins for purification of 13B, Häkan Svensson and Murthy Vrudhula for helpful discussions on both the work and the manuscript, Ana Wieman for manuscript preparation, and Drs. K. E. and I. Hellström for support and encouragement during the studies.

**REFERENCES**

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