Yeast Cytosine Deaminase Improves Radiosensitization and Bystander Effect by 5-Fluorocytosine of Human Colorectal Cancer Xenografts

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ABSTRACT

The efficacy of cancer gene therapy using bacterial cytosine deaminase (bCD)/5-fluorocytosine (5-FC) enzyme/prodrug strategy is limited by the inefficiency of cytosine deaminase (CD)-catalyzed conversion of 5-FC into 5-fluorouracil (5-FU). We have shown previously that yeast CD (yCD) is more efficient at the conversion of 5-FC than bCD. In the current study, we hypothesized that the increased production of 5-FU by yCD would enhance the efficacy of the CD/5-FC treatment strategy by increasing the bystander effect as well as the efficacy of radiotherapy because of the radiosensitizing capacity of 5-FU. To test this hypothesis, we generated stable HT29 human colon cancer cell lines expressing either bCD (HT29/bCD) or yCD (HT29/yCD). The amount of 5-FU produced in HT29/yCD tumors after a single injection of 5-FC (1000 mg/kg, i.p.) was 15-fold higher than that produced in HT29/bCD tumors. In tumor-bearing nude mice, the average maximum relative tumor size (compared with pretreatment values) of HT29/bCD tumors treated with 5-FC and irradiation (500 mg/kg i.p. and 3 Gy, 5 days a week for 2 weeks) was 0.55 ± 0.1, compared with 0.01 ± 0.01 in HT29/yCD tumors (P = 0.002). Moreover, an increased cytotoxic and radiosensitizing effect of 5-FC on bystander cells was observed in vitro and in vivo when yCD was expressed in HT29 cells instead of bCD. In mice bearing HT29 tumors containing 10% HT29/yCD cells, the combined treatment resulted in a minimum tumor size of 0.20 ± 0.07 compared with 0.60 ± 0.1 in 10% HT29/bCD cells (P < 0.001). These results demonstrate that the use of yCD in the CD/5-FC strategy has a high potential to improve the therapeutic outcome of combined gene therapy and radiotherapy in cancer patients.

INTRODUCTION

In enzyme/prodrug gene therapy, tumor cells are transduced with a gene encoding for a nonmammalian enzyme that converts a nontoxic prodrug into a cytotoxic and/or radiosensitizing drug. The local production of the toxic agent reduces systemic toxicity, which limits the efficacy of conventional chemotherapy.

The two most widely studied enzymes in the enzyme/prodrug strategy are HSV-TK-1 and bCD. HSV-TK converts GCV into a phosphorylated toxic metabolite (GCV-P) that inhibits DNA polymerase, resulting in DNA chain termination. CD deaminates the prodrug 5-FC into the cytotoxic and radiosensitizing agent 5-FU (1, 2). 5-FU is further metabolized to FdUMP, which inhibits thymidylate synthase resulting in depletion of TTP pools and eventually inhibition of DNA synthesis.

The CD/5-FC strategy has several potential advantages over the HSV-TK/GCV strategy when used for colon cancer treatment. First, the produced 5-FU is the chemotherapeutic agent of choice for treatment of colon cancer. Second, the CD/5-FC strategy appears to have a greater bystander effect. In contrast to GCV-P, 5-FU does not require gap junctional communication to kill nontransduced neighboring tumor cells (3). This is a major advantage, as gap junctions are often lost in cancer cells (4, 5). Third, 5-FU is not only cytotoxic, but also has radiosensitizing properties (6). Indeed, several in vitro and in vivo studies have now demonstrated that the CD/5-FC strategy is more effective than the HSV-TK/GCV system in lung and colon cancer cells (7, 8).

Although the CD/5-FC strategy holds promise, a severe limitation to its effectiveness has come from the fact that 5-FC is poorly deaminated by bCD (9). An enhancement of the conversion efficiency for 5-FC would be expected to substantially improve the efficacy of the CD/5-FC strategy both as a cytotoxic and radiosensitizing treatment. To this end, we have purified and characterized yCD, which has a 22-fold lower Kₘ for 5-FC compared with bCD. We have demonstrated that the use of yCD significantly improves the efficacy of the CD/5-FC treatment strategy in human colorectal cancer cells in vitro and in vivo (10).

In the present study, we have extended our comparison of bCD and yCD to radiosensitization by 5-FC in CD-transduced human colon cancer HT29 cells in vitro and in vivo. In addition, we have analyzed the bystander effect of yCD- and bCD-transduced HT29 cells, as well as the radiosensitizing effect of 5-FC on bystander cells. Our results demonstrate that yCD produces greater radiosensitization and an increased bystander effect compared with bCD. yCD has, therefore, a high potential to improve the therapeutic outcome of the combined enzyme/prodrug strategy and radiotherapy in cancer patients.

MATERIALS AND METHODS

Vectors and Cell Lines. The entire coding sequence of bCD, including an extra Kozak sequence followed by an ATG codon, was isolated by PCR (3). The yCD coding sequence (11), with modifications to facilitate expression in mammalian cells, was kindly provided by Gary Nolan, Stanford, CA).

HSV-TK/GCV Strategy. The human colon cancer cell line HT29 was grown as described previously (12). Stable HT29 cell lines expressing either bCD or yCD were generated by viral infection (10) using the retroviral expression vector Lazarus (kindly provided by Gary Nolan, Stanford, CA).

Cell Survival Assay. The radiosensitizing effect of 5-FC and 5-FU in HT29, HT29/bCD, and HT29/yCD cells was determined using a standard clonogenic assay (13). Cells were treated with 5-FC or 5-FU at various concentrations for 24 h before irradiation at 37°C in media containing 10% dialyzed serum. The radiation survival data were corrected for plating efficiency using a nonirradiated plate treated with 5-FC or 5-FU under the same conditions. The surviving fraction was plotted against the radiation dose, and curves were fit using the linear-quadratic equation. The radiation sensitivity was expressed as the MID, which represents the area under the cell survival curve (14). Radiosensitization was expressed as the ER, which was defined as ER = MIDcontrol /MID treated.

To determine the cytotoxic and radiosensitizing effect of 5-FC and 5-FU on bystander cells, cocultures of 90% bystander hygromycin-resistant HT29 cells and 10% puromycin-resistant CD-transduced HT29 cells were used. Cell
survival of the hygromycin-resistant HT29 cells and puromycin-resistant CD-
transduced HT29/cells was determined by plating the cells in selective media
after treatment and assessed using a standard clonogenic assay as described above.

**In Vivo 19F-MRS.** In vivo conversion of 5-FC to 5-FU in tumors was
monitored by 19F-MRS. Mice bearing s.c. tumors in the rear limb were injected
i.p. with 1000 mg/kg 5-FC and restrained on a specially constructed plastic jig
(15) to allow positioning of the tumor under a 6.1-mm single-turn surface coil.
Spectra were performed at 282.3 MHz on a Varian Magnetic Resonance
System equipped with an 18.3-cm horizontal bore 7.0 Tesla magnet. Spectra
were acquired as the average of 277 transients (free induction decays) collected
in 8K data points using a 25-µs pulse width (corresponding to a 90° flip angle
at a depth of 2.7 mm), a 4.328-ms repetition time, and a 25-KHz spectral
width; 20,000 scans were required for each spectrum. Metabolite concentrations
were calculated by normalizing their resonance-peak areas to that of a NaF
chemical shift and concentration standard in a microcell placed above the coil.
A scaling factor relating the NaF reference signal to 19F signals arising from
the tumor volume was obtained from MRS experiments using the NaF external
standard and a tumor phantom containing a known 5-FC concentration.

**GC/MS.** For the determination of 5-FC and 5-FU levels in plasma, tumors,
and normal organs by GC/MS, tumor-bearing mice were injected i.p. with
1000 mg/kg 5-FC. After 90 min, mice were sacrificed, and plasma, tumors,
liver, colon and muscle were collected and snap-frozen. Frozen tissue was
pulverized and weighed, and homogenates were made in 1 ml of 1 M acetic
acid using a polytron. Subsequently, samples were prepared for GC/MS as
described previously (16). Quantification of the derivatized products was
performed using a Varian Saturn 2000 GC/MS spectrophotograph in selected
monitoring mode. The amounts of 5-FC and 5-FU were expressed as µmol/g

**Mouse Model.** Nude female mice (Nu/Nu CD-1; Charles River Laborato-
ries, Wilmington, MA) 7–8 weeks of age were injected s.c. in the flank with
5 × 10^6 viable tumor cells. Tumors were measured with calipers in 2 dimen-
sions. Tumor volumes were calculated using the formula: m/6 (length ×
width)^2. When tumors measured an average volume of 100–150 mm^3, treat-
ment was started. Mice were treated 5 days a week for 2 weeks with either
5-FC (500 mg/kg i.p.), radiation (3 Gy), or a combination of both in which
5-FC was injected 3 h before radiation. The results were plotted as the average
tumor volume (in which a cured tumor was included as a tumor of zero size)
relative to that at the start of treatment versus time after initiation of the
treatment. Differences in the efficacy between treatments were expressed as
the number of tumor cures, defined as tumors that did not recur for 60 days,
and as the average minimum relative tumor volume for a given condition. Mice
were handled according to the established procedures of the University of

**Irradiation Conditions.** Cells were irradiated at room temperature at 1–2
Gy/min using an AECL Theratron 80 (60Co). Dosimetry was carried out using
a Baldwin ionization chamber connected to an electrometer system that was
directly traceable to a National Bureau of Standards calibration (12).

Tumor-bearing mice were restrained in a Lucite restrainer and placed
beneath the primary collimator and a secondary heavy alloy collimating device
on the (60Co) teletherapy unit. Mice were positioned such that the apex of
the tumor was at the center of a 2.4-cm aperture in the secondary collimator
(17).

**Immunohistochemistry.** Sections (5 µm thick) of frozen tumor tissues
were cut and mounted on slides (Fisher Scientific, Pittsburgh, PA). Tissues
were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Wash-
ington, PA) for 10 min at room temperature. Endogenous peroxidase activity
was blocked with 0.6% hydrogen peroxide in 100% methanol for 15 min at
room temperature. After the slides were washed in water, nonspecific binding
sites were blocked with 0.6% hydrogen peroxide in PBS for 15 min at
room temperature. Endogenous peroxidase activity was blocked with 10% normal goat serum PBS for 30 min at room temperature. Polyclonal anti-yCD and anti-bCD were generated in rabbits by

**RESULTS**

**Radiosensitizing Effect of 5-FC and 5-FU on HT29/bCD and
HT29/yCD Cells.** We have reported previously that HT29/yCD cells
were significantly more efficient at converting 5-FC to 5-FU than
HT29/bCD cells, resulting in a greater cytotoxicity (10). We then
hypothesized that HT29/yCD cells would be better radiosensitized by
5-FC. To study the effect of 5-FC on the radiation response, we
exposed HT29/bCD and HT29/yCD cells for 24 h to varying concentra-
tions of 5-FC, irradiated them, and determined the surviving frac-
tion. As we had hypothesized, the radiosensitizing effect of 5-FC was
significantly greater in HT29/yCD cells than in HT29/bCD cells (Fig.
1). At nontoxic concentrations, a ~3-fold lower concentration of 5-FC
was required to radiosensitize HT29/yCD cells to a similar extent as
HT29/bCD cells (P < 0.02).

To determine whether the difference in the radiosensitizing effect
of 5-FC in HT29/bCD and HT29/yCD cells was attributable to a

![Fig. 1. A, the effect of 5-FC on the radiation response of HT29/bCD and HT29/yCD
cells. Cells were exposed to 5-FC at 0 µM (□), 10 µM (■), or 100 µM (●) for 24 h before
radiation. Cells were then irradiated at various doses and assessed for clonogenicity. The
average values of surviving fractions of 3–4 experiments (± SE) are shown. B, the
radiosensitizing effect of 5-FC in HT29/bCD (open bars) and HT29/yCD (closed bars)
cells. Cells were treated and assessed as described in Fig. 1A. The radiosensitiveness is
expressed as the ER, which is calculated as MID_<sub>control</sub>/MID_<sub>treated</sub>.

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difference in sensitivity to 5-FU (as opposed to an increased 5-FU production), we also studied the effect of 5-FC on the radiation response of both cell lines. At the nontoxic concentration of 3 μM, both cell lines were radiosensitized by 5-FU, with a slightly higher sensitization of HT29/bCD cells (Fig. 2). At 10 μM, both cell lines were radiosensitized by 5-FU to a similar extent. Additional control experiments showed that HT29/bCD cells were more sensitive than HT29/yCD cells to radiation alone (MID of 2.51 ± 0.19 Gy and 3.74 ± 0.35 Gy, respectively). These results show that the enhanced radiosensitivity produced by 5-FC in HT29/yCD cells was not attributable to a greater sensitivity to radiation or 5-FU.

Cytotoxic and Radiosensitizing Effect of 5-FC on Bystander Cells. We then investigated the bystander effect of 5-FC in HT29/bCD and HT29/yCD cells in response to 5-FC treatment. Cocultures consisting of 90% bystander (hygromycin-resistant) HT29 cells and 10% CD-transduced (puromycin-resistant) HT29 cells were incubated with 300 or 1000 μM 5-FC for 24 h, and the surviving fraction of bystander HT29 cells was determined by plating the cells in hygromycin-containing media (Fig. 3A). For HT29/bCD cells, a small bystander effect was observed when cocultures were treated with 300 μM 5-FC. Increasing the concentration of 5-FC to 1000 μM did not significantly decrease the surviving fraction of bystander cells. In contrast, a concentration-dependent bystander effect was observed for HT29/yCD cells when cells were exposed to 5-FC, which was significantly greater than that observed for HT29/bCD cells (P < 0.001).

We next studied the radiosensitizing effect of 5-FC on bystander cells (Fig. 3B). Cocultures of 90% bystander and 10% CD-transduced HT29 cells were treated with 300 or 1000 μM 5-FC for 24 h and subsequently irradiated. No significant radiosensitization was produced in bystander cells after exposure to 300 μM 5-FC when cells were cocultured with HT29/bCD cells, whereas exposure to 1000 μM caused an ER of 1.6 ± 0.2 in bystander cells. When bystander cells were exposed to 300 μM 5-FC in the presence of HT29/yCD cells, significant radiosensitization was produced in bystander cells when compared with HT29/bCD cells (P < 0.01). Increasing the concentration of 5-FC to 1000 μM further increased the radiosensitivity of bystander cells.

In Vivo Monitoring of the Conversion of 5-FC to 5-FU. As the in vitro data provided strong evidence for the superior radiosensitizing effect of 5-FC in HT29/yCD cells, we decided to compare the effect of 5-FC on the radiation sensitivity of HT29/bCD and HT29/yCD tumors grown in nude mice. To obtain a maximum radiosensitizing effect by 5-FC, we first determined the in vivo time course of the radiosensitizing metabolite FdUMP after 5-FC injection. Mice bearing HT29/yCD tumors were injected i.p. with 1000 mg/kg 5-FC and the levels of 5-FC, 5-FU, and metabolites were monitored by 19F-MRS (Fig. 4A). Peak levels for 5-FC and 5-FU were observed 60 and 90 min after injection, respectively, and rapidly declined thereafter with half lives of 90 ± 9 (5-FC) and 75 ± 6 (5-FU) min. FdUMP, of which FdUMP is the major component, was first observed 60 min after injection, reached a steady state approximately 160 min after injection, and remained at that level for at least an additional 4 h.

In addition to HT29/yCD tumors, we also examined the effects of 5-FC, 5-FU, and the metabolites in HT29/bCD and HT29 tumors by 19F-MRS at 100 min after injection of 1000 mg/kg 5-FC (Fig. 4B). In contrast to HT29/yCD tumors, in which all compounds could be detected, only 5-FC was measurable in HT29/bCD and HT29 control tumors.

To determine whether the absence of the 5-FU peak in HT29/bCD tumors was attributable to a limitation of the detection sensitivity of 19F-MRS, we also measured 5-FC and 5-FU levels in tumor tissue by GC/MS analysis (Fig. 4C). Tumor-bearing mice were injected with 1000 mg/kg 5-FC, and 90 min after injection, mice were sacrificed. Plasma, tumors, liver, colon, and muscle were prepared for analysis by GC/MS. For both HT29/bCD and HT29/yCD tumors, 5-FU could be detected, but the concentration of 5-FU in HT29/yCD tumors was approximately 15-fold higher than that in HT29/bCD tumors. The measured amount of 5-FC in HT29/yCD tumors was in correspondence with that observed by 19F-MRS. In control HT29 tumors, only 5-FC was present.

Radiosensitizing Effect of 5-FC in HT29/bCD and HT29/yCD Tumors. To study the radiosensitizing effect of 5-FC in vivo, mice bearing HT29/bCD or HT29/yCD tumors were treated with either 5-FC, irradiation, or a combination of both treatment modalities. On the basis of the kinetic data obtained by 19F-MRS, tumors were irradiated 3 h after 5-FC administration at the time of maximum FdUMP levels in the tumors (see above).

In HT29/bCD tumors, 5-FC treatment caused a small growth delay,
and no regressions were observed (Fig. 5). Tumors treated with either radiation or a combination of 5-FC and radiation showed regression, but most regrew after day 30. Tumor cures were observed in 3 of 9 irradiated tumors and in 1 of 10 tumors treated with a combination of 5-FC and radiation. The addition of 5-FC to radiotherapy did not appear to result in a better therapeutic effect in HT29/bCD tumors. Treatment with 5-FC produced significantly better results in HT29/yCD tumors compared with HT29/bCD tumors \( (P < 0.005) \). Tumor regressions were observed, and 8 of 16 tumors were cured (Fig. 5). When HT29/yCD tumors were treated with a combination of 5-FC and radiation, the therapeutic effect significantly improved compared with radiation alone \( (P < 0.005) \), with seven of nine tumors being cured. We also assessed the effect of 5-FC and radiation on the relative tumor size. We found that the minimum relative tumor size (compared with pretreatment values) of HT29/bCD tumors treated with 5-FC and radiation was 0.55 \( \pm 0.1 \), compared with 0.01 \( \pm 0.01 \) in HT29/yCD tumors \( (P = 0.002) \). This improvement was not attributable to a greater sensitivity of HT29/yCD tumors to radiation alone, because their radiosensitivity was similar to that of HT29/bCD tumors (minimum relative tumor volume 0.61 \( \pm 0.21 \) and 0.65 \( \pm 0.19 \)). These findings confirmed our hypothesis that yCD would produce a greater antitumor effect than bCD when combined with radiation.

**Fig. 4.** A, in vivo monitoring of the conversion of 5-FC into 5-FU in HT29/yCD tumors by \(^{31}P\)-MRS. Tumor-bearing mice were injected i.p. with 1000 mg/kg 5-FC and the kinetics of 5-FC (○), 5-FU (●), and FNuc (▲) were monitored. Each point is the average of five animals. The indicated times mark the start of the acquisition period ± 10 min. B, representative in vivo \(^{31}P\)-MRS spectra of HT29, HT29/bCD, and HT29/yCD tumors at 100 min after injection with 1000 mg/kg 5-FC. 1, FNuc (−45 ppm); 2, 5-FC (−48.5 ppm); 3, 5-FU (−49.6 ppm). C, determination of 5-FC (left) and 5-FU (right) levels in plasma, tumors, liver, colon, and muscle in mice bearing HT29/bCD (open bars), HT29/yCD (closed bars), or HT29 (hatched bars) tumors by GC/MS. Tumor-bearing mice were injected i.p. with 1000 mg/kg 5-FC. Ninety min after injection, plasma, tumors, and organs were collected and processed for GC/MS. The measured amount of 5-FC and 5-FU is expressed as \( \mu g/\text{g tissue} \). The average values of 4–8 samples (± SE) are shown.

**Fig. 5.** Growth of HT29/bCD (top) and HT29/yCD (bottom) tumors in response to either 5-FC treatment (500 mg/kg; ○), radiation (3 Gy; ◆) or a combination of 5-FC and radiation (▲). Treatment was given daily at days 0–4 and 7–11, and the time interval between 5-FC and radiation in the combined treatment was 3 h. The average volumes (± SE) relative to those at day 0 of control tumors (●; \( n = 12–20 \)) and treated tumors (\( n = 9–16 \)) are shown.

As current gene-delivery systems do not transduce all tumor cells, the therapeutic outcome of the CD/5-FC treatment strategy in combination with radiotherapy is largely dependent on the radiosensitizing effect of 5-FU on bystander cells. To study the effect of 5-FC on the radiation response of bystander cells in vivo, we treated mice with tumors consisting of a mixture of 90% bystander and 10% CD-transduced HT29 cells with 5-FC, radiation, or a combination of both modalities, as described above.

Mixed tumors containing HT29/bCD cells did not respond to 5-FC treatment alone (Fig. 6). In addition, consistent with tumors derived entirely from HT29/bCD cells, mixed tumors did not show an im-

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proved therapeutic outcome when 5-FC was combined with radiation. Tumor regression was observed with a regrowth after day 35, but no tumor cures were observed. In tumors containing HT29/yCD cells, treatment with 5-FC caused a tumor growth delay, but no regressions were observed. However, the addition of 5-FC to radiation resulted in significantly more tumor regressions when compared with radiation alone, and 6 of 11 tumors were cured ($P < 0.025$). Similarly, in mice bearing HT29 tumors containing 10% HT29/yCD cells, the combined treatment resulted in a minimum relative tumor size of $0.20 \pm 0.07$ compared with $0.60 \pm 0.1$ in 10% HT29/bCD cells ($P < 0.001$). These results show that 5-FC had neither cytotoxic nor radiosensitizing effects on bystander tumor cells in the presence of HT29/bCD cells, whereas a small cytotoxic effect and a significant radiosensitizing effect of 5-FC were observed in bystander cells in the presence of HT29/yCD cells.

A possible explanation for the greater bystander effect observed in tumors containing yCD cells compared with bCD cells was that yCD cells grew more rapidly in the mixed tumor than bCD cells. To test this possibility, animals were prepared bearing tumors that were 0%, 10%, or 100% HT29/yCD cells or HT29/bCD cells using techniques identical to those described for the therapy experiments. We then assessed the fraction of cells that were CD-positive using immunohistochemistry for bCD and for yCD (Fig. 7). We found that the fraction of positively staining cells in the 10% condition was equal for both the yCD and bCD mixed tumors (although the actual fraction that stained positive was 14%, suggesting that both HT29/yCD cells and HT29/bCD cells may grow faster than bystander cells; Table 1). Thus, the different responses between mixed tumors of bystander cells with either HT29/yCD or HT29/bCD cells is not attributable to a difference in the percentage of CD-expressing cells at the time of 5-FC with or without radiation.

**DISCUSSION**

Our results show that, compared with bCD, yCD expression significantly improves the efficacy of combined 5-FC and radiation therapy in human colon cancer xenografts. This improvement can be explained by the higher conversion efficiency of 5-FC to 5-FU by yCD. Moreover, the use of yCD also resulted in greater cytotoxicity to and radiosensitization of bystander tumor cells. As only 5–10% of the tumor cells are transduced with current gene-delivery systems, our

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**Fig. 6. Growth of mixed tumors, consisting of 90% bystander HT29 cells and 10% HT29/bCD cells (top) or 10% HT29/yCD cells (bottom) in response to 5-FC treatment (500 mg/kg, ○), radiation (3 Gy, □), or a combination of 5-FC and radiation (■). Treatment was given daily at days 0–4 and 7–11, and the time interval between 5-FC and radiation in the combination treatment was 3 h. The average volumes (± SE) relative to those at day 0 of control tumors (●, $n = 4–6$) and treated tumors ($n = 4–11$) are shown.**

**Fig. 7. Estimation of fraction of CD-containing cells in mixed tumors. Animals bearing tumors that were 0% (A), 10% (B), or 100% (C) HT29/yCD cells or 0% (D), 10% (E), or 100% (F) HT29/bCD cells at the time of inoculation were prepared using techniques identical to those described for the therapy experiments. When tumors attained the appropriate size, they were prepared for immunohistochemistry as described in “Materials and Methods.” A representative field for each condition is shown (×500).**
Table 1 Fraction of CD-containing cells in mixed tumors assessed by immunohistochemistry

<table>
<thead>
<tr>
<th>Condition</th>
<th>Antibody</th>
<th>Number positive/total number</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bystander</td>
<td>Anti-bCD</td>
<td>0/100</td>
<td>0</td>
</tr>
<tr>
<td>10% HT29/bCD</td>
<td>Anti-bCD</td>
<td>61/420</td>
<td>14.5</td>
</tr>
<tr>
<td>100% HT29/bCD</td>
<td>Anti-bCD</td>
<td>129/160</td>
<td>80.6</td>
</tr>
<tr>
<td>Bystander</td>
<td>Anti-yCD</td>
<td>0/100</td>
<td>0</td>
</tr>
<tr>
<td>10% HT29/yCD</td>
<td>Anti-yCD</td>
<td>239/1670</td>
<td>14.3</td>
</tr>
<tr>
<td>100% HT29/yCD</td>
<td>Anti-yCD</td>
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findings indicate that yCD is better suited than bCD for cancer gene therapy. This conclusion is strengthened by the fact that the dose-limiting toxicity for 5-FC in humans is attributable to 5-FU production by *Escherichia coli* in the gut, and that the *K*₅₀ of yCD for 5-FU is 22-fold lower than that of *E. coli* CD.

In a previous study we reported on the preferential killing of bCD-expressing tumor cells in response to 5-FU treatment compared with bystander cells, because of the high intracellular 5-FU concentration (3). It was possible that the higher conversion efficiency of HT29/yCD cells for 5-FC, resulting in a higher intracellular concentration of 5-FU, could have killed the "factory" prematurely, thereby reducing the bystander effect of HT29/yCD cells. Our *in vitro* data showed, however, an increased bystander effect of HT29/yCD cells when compared with HT29/bCD cells, suggesting that the rapid and substantial 5-FU production was sufficient to kill both yCD-transduced producer cells and bystander cells. Nevertheless, temporarily sparing CD-transduced tumor cells from cytotoxicity by intracellular 5-FU might result in a further increase in bystander effect as more 5-FU can ultimately be produced. To increase temporarily the life span of transduced cells, previously we generated a secreted form of bCD (2). We found that tumor cells that expressed secreted bCD survived longer and produced a higher extracellular concentration of 5-FU in response to 5-FU treatment than cells that expressed intracellular bCD. Thus, we would anticipate that the use of a secreted form of yCD, which is currently being generated, might be even more effective than the intracellular enzyme.

Studies have reported on the radiosensitizing effect of 5-FU in tumor cells transduced with bacterial CD *in vitro* (18) as well as *in vivo* (19). A maximal radiosensitizing effect of 5-FU in nude mice bearing WiDr human colon cancer xenografts transduced with bCD was observed after treatment with 5-FC for 6 consecutive days followed by a single radiation dose (19). This is in contrast to our results with HT29/bCD tumors, as we did not observe a radiosensitizing effect of 5-FC. The improved efficacy of the combined treatment in WiDr/bCD tumors might be explained by a possible higher expression of bCD in WiDr cells resulting in a greater sensitivity to 5-FC. Indeed, treatment of WiDr/bCD tumors with 5-FC alone did result in a tumor growth delay, whereas we observed almost no growth delay in HT29/bCD tumors in response to 5-FC treatment. Gabel et al. (19) also showed that the combined treatment of 5-FC and radiation was more effective in tumor-growth inhibition than a similar treatment of the conventional combined treatment of 5-FU and radiation. By using yCD instead of bCD, this difference in efficacy is expected to be higher, which may validate the preferential use of the CD/5-FC strategy over 5-FU when combined with radiotherapy in cancer patients.

Another approach to increase the radiosensitivity of tumors is to combine radiotherapy with double suicide gene therapy, in which tumor cells are transduced with a fusion protein of CD and HSV-1 TK (20). This combined treatment potentiated the antitumor effect when compared with that of the single treatment modalities alone. However, the efficacy of this approach in tumors containing only a small percentage of transduced cells (as will be the case in patients) remains to be determined, because the radiosensitizing and cytotoxic effect of the HSV-TK/GCV on bystander tumor cells is dependent on gap junctional communication, which is often eliminated in tumors.

The first *in vivo* studies of radiosensitization by 5-FC in adenovirally infected tumors have been published recently (21, 22). In mice bearing human squamous cell carcinoma xenografts (SQ-20B), adenoviral-directed bacterial CD/5-FU gene therapy did enhance the efficacy of fractionated radiotherapy in small and large tumors (21). No tumor cures were observed in large tumors in response to the combined treatment, whereas three of seven small tumors were cured. Because this tumor type was resistant to 5-FU, the improved efficacy was considered to be attributable to an enhanced radiosensitization. In another study (22), human cholangiocarcinoma xenografts (SK-ChA-1) infected with an adenovirus encoding for bCD also showed an improved response to the combined treatment of 5-FC and radiation. On the basis of the current study, the use of yCD would be expected to improve the therapeutic outcome of this treatment strategy in virally infected tumors.

One limitation of our study is that we used athymic nude mice bearing human tumor xenografts. Although this immunocompromised model is required to grow human tumors, it is possible that the immune system could ultimately play a role in tumor response, particularly because CD is not a mammalian enzyme. Additional experiments in an immunocompetent host will be required to address this issue in the future.

In conclusion, our results show an improved therapeutic effect of the combined treatment with 5-FC and radiation when tumor cells are transduced with yCD instead of bCD. This suggests that the combination of radiotherapy and the yCD/5-FC strategy has a high potential to increase the efficacy of radiation in cancer patients. Because the results in mixed tumors were promising, we are currently optimizing adenovirus-mediated delivery of yCD to tumors in a preclinical model of colorectal cancer.

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Yeast Cytosine Deaminase Improves Radiosensitization and Bystander Effect by 5-Fluorocytosine of Human Colorectal Cancer Xenografts

Els Kievit, Mukesh K. Nyati, Emily Ng, et al.

_Cancer Res_ 2000;60:6649-6655.

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