Cytosine Deaminase/5-Fluorocytosine-Based Vaccination Against Liver Tumors: Evidence of Distant Bystander Effect

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Background: The cytosine deaminase gene of Escherichia coli converts the nontoxic compound 5-fluorocytosine into 5-fluorouracil (5-FU), thereby acting as a suicide gene when introduced into cancer cells, killing the cells when they are exposed to 5-fluorocytosine. We analyzed the efficacy of using cytosine deaminase-bearing cancer cells as an autologous tumor vaccine in a rat model that mimics liver metastasis from colon carcinoma.

Methods: We introduced a plasmid vector containing the E. coli cytosine deaminase gene into a BDIX rat colon carcinoma cell line. Intrahepatic injection of the modified cells in syngeneic animals generates a single experimental liver “suicide tumor.” We then analyzed the effect of 5-fluorocytosine treatment in terms of regression of cytosine deaminase-expressing cells in vivo as well as protection against wild-type cancer cells.

Results: Treatment with 5-fluorocytosine induced regression of cytosine deaminase-expressing (CD+) tumors, with seven of 11 treated animals being tumor free at the end of 30 days and a statistically significant difference in tumor volumes between treated and control animals (two-sided $P<0.0001$). Intrahepatic injection of CD+ cells followed by 5-fluorocytosine treatment rendered the treated animals resistant to challenge with wild-type tumor cells, with no (zero of seven) treated animals developing wild-type tumors in contrast to all (four of four) control animals. Moreover, in animals with established wild-type liver tumors, injection of CD+ tumor cells followed by 5-fluorocytosine treatment produced a statistically significant increase in survival time (two-sided $P<0.0001$). In vivo immunodepletion and immunohistologic analysis of experimental tumors indicate that natural killer cells are the major immune component involved in this antitumor effect.

Conclusions and Implications: Taken together, these results suggest the potential use of suicide gene-modified tumor cells as therapeutic vaccines against liver metastasis from colon carcinoma. [J Natl Cancer Inst 1999;91:2014–9]

The high lethality of colon carcinoma is due in part to the appearance of liver metastasis in the 30%–40% of patients for whom surgery and chemotherapy treatments appeared to be ineffective (1,2). Gene therapy constitutes a promising alternative for the treatment of cancer. Among the different approaches, suicide gene therapy—which consists of the transfer into tumor cells of a “killer gene” that activates a prodrug to its cytotoxic form—seems to be a particularly suitable strategy because it is applicable to all varieties of tumors. Two types of suicide systems have been studied in particular: 1) the herpes simplex virus thymidine kinase gene (HSV-tk) with ganciclovir (GCV) as the prodrug and 2) the cytosine deaminase gene (CD) of Escherichia coli, which converts the nontoxic antifungal agent 5-fluorocytosine into 5-fluorouracil (5-FU). To date, the CD gene has been introduced by transfection or transduction into different types of tumor cells, including colon carcinoma (3–5), fibrosarcoma (3), and mammary adenocarcinoma (6). In these cases, the suicide gene-bearing tumor cells were implanted subcutaneously in mice, and treatment with 5-fluorocytosine induced a substantial regression of the cytosine deaminase-expressing (CD+) tumor. In addition, this treatment...
resulted in the induction of a protective immunity against subcutaneously injected wild-type tumor cells.

In this study, we analyzed, in a model system in which colon carcinoma cells were engrafted in the liver of rats to mimic formation of a metastatic tumor, the efficacy of vaccination with tumor cells modified to express the CD gene.

**MATERIALS AND METHODS**

**Plasmid**

The CD gene was excised from the pCD2 plasmid (7) (EcoRI–BamHI) and cloned in the NotI site of pDNAβgeo vector (constructed by V. Pierrefitte-Carle, unpublished data). The resulting construct, designated pCDβgeo, contains the CD gene under the control of the cytomegalovirus promoter and a fusion gene between neo and lacZ, located downstream of the PGK (phosphoglycerate kinase) promoter.

**Antibodies**

Mouse monoclonal antibodies against B lymphocytes (anti-CD45 RA, OX-33; Pharmingen, San Diego, CA), macrophages (anti-CD11b/c, OX-42; Pharmingen), natural killer (NK) cells (NKR-P1, 10-78; Cedarian, Ontario, Canada), and T lymphocytes (anti-CD2, MRC OX-34; anti-CD8, MRC OX-8; anti-CD4, W3/25; Cedarian) were used for fluorescence-activated cell sorting and immunohistologic analyses. Labeling was performed with fluoro-oroxytoxycyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dako, Trappes, France). Rabbit anti-asialo GM1 antibodies were used to deplete NK cells in vivo (Wako Chemicals GmbH, Neuss, Germany).

**Cell Culture and Transfection**

DHD/K12/PROb (PROb) cells constitute a colon carcinoma cell line originating from a chemically induced colon cancer in BDIX rats (8). These cells are poorly immunogenic and induce progressive and metastatic tumors in syngeneic hosts (9). The cells were maintained in Dulbecco’s modified Eagle medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% fetal calf serum. For transfection experiments, cells were trypsinized, washed, and resuspended at 10^7 cells/mL in cold phosphate-buffered saline. Then 400 μL of this cell suspension was incubated on ice for 10 minutes in the presence of 5 μg of plasmid DNA and 15 μg of salmon sperm DNA used as a carrier. The reaction mixture was subjected to six 99-μsec electric pulses at 960 V (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) and placed on ice for 10 minutes, and the cells were plated in four 75-cm^2 culture flasks. Two days later, transfected cells were selected by the addition of G418 (Sigma, L’Isle D’Abeau Chesnes, France) at 100 μg/mL to standard medium.

**Experimental Protocols**

For all of the experiments, we used adult BDIX male rats weighing 180–250 g (IFFA CREDO, L’Arbresle, France). All of the surgical procedures and the care given to the animals were in accordance with institutional guidelines. All of the animals were randomly assigned to treatment.

**Induction of single liver tumor and evaluation of antitumor immunity.** At day 0, the rats had surgery, and 1.5 × 10^6 PRObCD tumor cells were injected under the liver capsule in the right lobe. Treatment with 5-fluorocytosine began at day 1. After 30 days of treatment, all animals had surgery, and the CD+ tumor, if any, was removed from the right lobe. During the same surgical procedure, 1.5 × 10^6 parental PROb cells were injected under the liver capsule in the left lobe in some of the animals. All of the rats were killed 12 days later and analyzed for the presence of a tumor.

**Effect of suicide cell-based vaccination against a wild-type pre-existing tumor.** A total of 1.5 × 10^6 parental PROb cells were injected under the liver capsule in the right lobe. Five days later, the animals had surgery, they were checked for the presence of tumor, and 1.5 × 10^6 PRObCD cells were injected in the left lobe. After 24 hours, the rats were treated with 5-fluorocytosine for 30 days. At the end of the treatment, the rats were killed, and tumor volumes were measured. When the experiment was carried out in the presence of dexamethasone or anti-NK antibodies, the rats were killed after 15 days of treatment.

**Treatment with 5-fluorocytosine.** 5-Fluorocytosine (Roche, Fontenay Sous Bois, France) was dissolved in saline (15 mg/mL), and the rats received daily three intraperitoneal injections of the drug (800 mg/kg of body weight) for 30 days. In the survival experiment, this treatment was followed by a daily 5-fluorocytosine injection (5 days a week) for 3 months.

**Treatment with dexamethasone.** Dexamethasone (Merck Sharp & Dohme–Chibret, Paris, France) was injected intraperitoneally daily at 750 μg/kg of body weight for 15 days.

**Treatment with anti-NK cells antibodies.** We injected 250 μL of rabbit anti-asialo GM1 antibodies (23 mg/mL immunoglobulins) at days 1, 5, 10, and 14 of 5-fluorocytosine treatment. As a control, rabbit immunoglobulins (23 mg/mL) were injected on the same schedule in a separate group of rats.

**Statistical Analysis**

For animal experiments, the results are expressed as median (95% confidence interval [CI]; range = minimum–maximum), and tumor volumes were compared between treated and control rats by use of the Mann–Whitney test, which is a nonparametric, two-tailed probability test. Qualitative analysis (presence of a tumor) was performed by use of two-tailed Fisher’s test. For the survival experiment, we used Kaplan–Meier survival analysis. The logrank test was used to compare the survival of vaccinated and control animals. All statistics were computed with SPSS 8.0 software (SPSS Inc., Chicago, IL). P values were considered to be statistically significant when less than .05.

**RESULTS**

**Regression of CD+ Tumors Upon 5-Fluorocytosine Action In Vivo**

The pCDβgeo plasmid was introduced into the poorly immunogenic DHD/K12/PROb (PROb) rat cell line, generating PROb cells stably expressing CD (PRObCD). Prior to evaluation of the efficacy of our cell-based vaccine, we first analyzed the regression of PRObCD cells following 5-fluorocytosine action in vivo. Of eleven 5-fluorocytosine-treated animals, seven did not exhibit any liver tumors at the end of the treatment, and the four remaining rats each exhibited a residual tumor with a median volume of 0 mm^3 (95% CI = 0–2.5 mm^3; range = 0–12.5 mm^3) (Fig. 1). All control animals (n = 8) treated with physiologic saline exhibited a tumor, with a median volume of 40 mm^3 (95% CI = 13.3–63.6 mm^3); range = 3.5–131 mm^3) (P<.0001).

**Protective Immunity Induced After Regression of CD+ Tumors and Cure of Established Liver Tumors by Suicide-Cell-Based Vaccination**

To determine whether rats carrying a CD+ tumor treated with 5-fluorocytosine developed immunity to wild-type tumor,
we rechallenged some of the animals of the preceding experiments in the opposite liver lobe with parental PROb cells. After 12 days, we did not detect any tumor in any (seven of seven) treated animals, while all control rats (four of four) developed a tumor \( (P = .003) \) (data not shown). These results demonstrate that rats pretreated with CD+ tumor and 5-fluorocytosine exhibited resistance to a new intrahepatic challenge with wild-type tumor cells.

To further evaluate the efficacy of the observed antitumor effect, we investigated the effect of 5-fluorocytosine treatment of a PRObCD tumor on the development of a pre-existing wild-type tumor (Fig. 2, A). In the case of PRObCD tumors, although 11 of 11 rats in the control group exhibited a tumor with a median volume of 5 mm\(^3\) (95% CI = 3.8–66.7 mm\(^3\); range = 0.5–188 mm\(^3\)), only two of 10 treated rats had a tumor with a median volume of 0 mm\(^3\) (95% CI = 0–0.1 mm\(^3\); range = 0–9 mm\(^3\)) \( (P<.0001) \). The case PROb pre-existing tumors, all of the saline-treated animals \( (n = 11) \) carried a tumor, with a median volume of 102 mm\(^3\) (95% CI = 30.9–156.6 mm\(^3\); range = 6–205 mm\(^3\)). Among 5-fluorocytosine-treated rats \( (n = 10) \), four were tumor free after the treatment and six had residual tumors with volumes ranging from 0.006 to 8 mm\(^3\) (median = 0.1 mm\(^3\); 95% CI = 0–6.1 mm\(^3\)) \( (P<.001) \). These results indicate that a distant bystander effect can be generated by the suicide of PRObCD cells upon 5-fluorocytosine action and that this effect can act in a curative manner on pre-existing wild-type tumor.

**Involvement of NK Cells in the Distant Antitumor Effect**

Because 5-fluorocytosine was ineffective in PROb tumors (data not shown), the regression of parental tumors could be due to an immunologic reaction stimulated by the regression of PRObCD tumors or, alternatively, to the presence of substantial levels of plasma 5-FU produced by the PRObCD tumor. To test the latter hypothesis, we first analyzed systemic 5-FU concentration in 5-fluorocytosine-treated rats. No measurable 5-FU concentration (≤5 ng/mL) could be detected in the plasma of these treated rats, which supports the idea that an immune response can be responsible for the antitumor effect on wild-type tumor cells. We further investigated this hypothesis by repeating the vaccination of rats bearing a PROb pre-existing tumor in the presence of the immunosuppressor dexamethasone. We found that dexamethasone induced a dramatic decrease in the lymphocyte lineages (CD4+, CD8+, and CD45+), with the exception of NK cells, which were slightly affected or not affected by this treatment (data not shown). In addition, dexamethasone had no statistically significant inhibitory effect on the regression of both types of tumor, indicating that the distant bystander effect was roughly conserved, although the amount of circulating T lymphocytes was dramatically reduced (data not shown). Together, these results suggest that NK cells could be responsible for the distant bystander effect observed in our model.

For further assessment of this hypothesis, an immunodepletion of NK cells was achieved by injection of anti-asialo GM1 antibodies during the 5-fluorocytosine treatment. The results (Fig. 2, B) indicated that there was no statistically significant difference in tumor volumes of saline-treated rats (median, 16 mm\(^3\); 95% CI = 5.5–33.9 mm\(^3\); range = 4–42 mm\(^3\)) and 5-fluorocytosine/anti-NK-treated rats (median, 5.2 mm\(^3\); 95% CI = 1.3–57.0 mm\(^3\); range = 1.3–57.0 mm\(^3\)) \( (P = .42) \), whereas tumor volumes of saline-treated rats were markedly larger than those of 5-fluorocytosine-treated animals (median = 0.01 mm\(^3\); 95% CI = 0–0.5 mm\(^3\); range = 0–2 mm\(^3\)) and the difference was statistically significant \( (P = .001) \). The 5-fluorocytosine/immunoglobulin-treated rats exhibited tumor volumes (median = 1.0 mm\(^3\); 95% CI = 0–13.0 mm\(^3\); range = 0–13 mm\(^3\)) that were not statistically significantly different from those of 5-fluorocytosine-treated animals \( (P = .45) \). Tumor volumes of 5-fluorocytosine-treated rats were statistically significantly different from those of the 5-fluorocytosine/anti-NK-treated group \( (P = .005) \). Because of the presence of one larger tumor, the 5-fluorocytosine/immunoglobulin-treated rats exhibited tumor volumes that were not statistically significantly different from those of the 5-fluorocytosine/anti-NK-treated group \( (P = .08) \). Taken collectively, these data indicate that the administration of anti-NK antibodies markedly decreases the distant bystander effect observed in our model.

Consistent with these results, immunohistologic analysis of PROb and PRObCD tumors in rats treated with saline or 5-fluorocytosine showed a differential distribution of NK cells according to the treatment. As shown in Fig. 3, F, 5-fluorocytosine treatment was associated with an infiltration of NK cells within the
tumor (PROb or PRObCD), whereas NK cells were only found surrounding the tumors in saline-treated animals (Fig. 3, E).

T lymphocytes were located at the periphery of PROb and PRObCD tumors, regardless of the treatment (Fig. 3, D). In the case of 5-fluorocytosine/anti-NK-treated rats, no NK cells were observable in the liver sections (data not shown).

Increased Survival Rate of Tumor-Bearing Rats After Vaccination With Autologous Suicide Tumor Cells

We then analyzed the efficacy of the treatment on the survival of rats carrying a pre-existing liver tumor (Fig. 4). All control animals died between 61 and 135 days after the injection of PROb cells (median: 91 days). Five treated rats died between 130 and 154 days after the injection of PROb cells, and the five remaining animals were still alive at day 180 (median: 154 days). These results demonstrate that vaccination with suicide tumor cells increases the survival rate of rats carrying a liver tumor in a statistically significant manner ($P < .0001$).

**DISCUSSION**

We have used colon carcinoma cells that express the CD gene to test the efficacy of such cells in inhibiting the development of experimental liver metastases generated by intrahepatic injection of colon cancer cells. Administration of 5-fluorocytosine led to a 95% decrease in the mean tumor volume of CD+ tumors compared with controls and rendered the rats resistant to later challenge with parental tumor cells. Furthermore, this vaccination also appeared to induce a distant bystander effect, leading to the regression or cure of a pre-established parental liver tumor. Although a distant bystander effect has not been firmly established yet in the cytosine deaminase/5-fluorocytosine suicide system, regression of untransduced tumors growing at a distance from transduced tumors is well documented in the thymidine kinase/GCV system (12–16).

Several hypotheses have been proposed to explain the thymidine kinase-related distant antitumor effect. The involvement of the immune system was proposed in most studies, based on the presence of T-lymphocyte infiltrates within the tumors (12,14–16).

However, a distant bystander effect was also observed in immunodeficient SCID mice, which suggests that alternative mechanisms may be at work, such as the presence of a soluble factor that might contribute to this distant antitumor effect (13). In our studies, regression of wild-type tumor did not stem from the production of 5-FU by the PRObCD tumor on the opposite lobe of the liver, since 5-FU was not detectable in the plasma of 5-fluorocytosine-treated rats. Instead, the hypothesis of an immune mechanism triggered by the death of PRObCD tumor cells upon 5-fluorocytosine action seems more likely. To better delineate the role of the immune system in our model, we analyzed the influence of dexamethasone on the regression of a wild-type tumor after vaccination with PRObCD cells and 5-fluorocytosine treat-
ment. The results of this experiment suggest that NK cells may be involved in the distant bystander effect observed in our model. This hypothesis was further supported by NK cell immunodepletion in vivo, which showed that depletion of NK cells leads to the loss of the distant bystander effect. In addition, immunohistochemical observations demonstrated an infiltration of NK cells within the tumor, dependent on the 5-fluorocytosine treatment. These results are consistent with those of Hall et al. (17) who demonstrated that a potent antitumor NK cell activity is induced subsequent to HSV-tk transduction and GCV treatment in an orthotopic mouse model of prostate cancer. However, although the difference in tumor volumes between 5-fluorocytosine/anti-NK-treated rats and saline-treated animals was not statistically significant, volumes in the former group seem to be lower than those in the latter group. These observations suggest a possible involvement of a second, minor immune lineage—such as CD8+ T lymphocytes—in the regression of these distant tumors. Nevertheless, our results indicate that NK cells are the major immune component involved in the distant bystander effect observed in our model. In this regard, cytotoxic NK1.1 T cells, which display a double NK/T phenotype and exhibit CD45 RO+ memory status, appear as good candidates, which may reconcile the implication of NK antigen-expressing cells in tumor rejection and the evidence of an antitumor immune memory (18).

To determine the potential therapeutic benefit of vaccination with autologous suicide tumor cells, we performed a survival experiment. Our results demonstrate that this vaccination approach statistically significantly increases the survival rate of rats carrying a wild-type liver tumor, five of 10 vaccinated rats still being alive 6 months after the injection of PROb tumor cells. Importantly, no tumor was observed in the left liver lobe of four of the five treated rats that died, indicating that the vaccinating PRObCD cells injected in this lobe do not generally escape the 5-fluorocytosine treatment, even in long-term experiments.

Taken together, these data indicate that suicide cell-based vaccination could be a promising therapy to prevent or to cure disseminated liver metastasis from colon cancer.

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NOTES

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