Virally Directed Cytosine Deaminase/5-Fluorocytosine Gene Therapy Enhances Radiation Response in Human Cancer Xenografts

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Abstract

Gene therapy combined with radiation therapy to enhance selectively radiation cytotoxicity in malignant cells presents a new approach for cancer treatment. We investigated the efficacy of adenoviral (Ad5)-directed cytosine deaminase/5-fluorocytosine (CD/5-FC) enzyme/prodrug gene therapy to enhance selectively the tumoricidal action of ionizing radiation in human cancer xenografts derived from a human squamous carcinoma cell line (SQ-20B). Tumor xenografts grown in hindlimbs of nude mice were transfected with an adenoviral vector (Ad.CMV.CD) containing the cytosome deaminase (CD) gene under the control of a cytomegalovirus (CMV) promoter. Mice were injected i.p. with 800 mg/kg of 5-FC for 12 days, and tumors were treated with fractionated radiation at a dose of 5 Gy/day to a total dose of 50 Gy. In larger tumors with a mean volume of 1069 mm3, marked tumor regression to 11% of the original tumor volume was observed at day 21 (P = 0.01). The volumetric regression of smaller tumors with a mean volume of 159 mm3, which received the same combined treatment protocol, was significant at day 12 (P = 0.014). However, unlike large tumors, regression of the smaller tumors continued until day 36 (P = 0.01), with 43% cured at day 26. No cures or significant volumetric reduction in size was observed in tumors treated with radiation alone; Ad.CMV.CD with or without radiation; or with Ad.CMV.CD and 5-FC. These results suggest that the CD/5-FC gene therapy approach is an effective radiosensitizing strategy and may lead to substantial improvement in local tumor control that would translate into improved cure rates and better survival.

Introduction

Radiation therapy is frequently used as a primary or adjuvant treatment for many human cancers with curative intent or to improve local tumor control. For many large epithelial and mesenchymal tumors or tumors of certain histological types, such as glioblastoma, complete tumor eradication and local cure following radiotherapy are rare. Potential mechanisms of radiation resistance include a large population of clonogenic tumor cells, repopulation of tumor cells between doses, cellular repair of radiation damage, and inherent tumor radioresistance (1). Also microenvironmental factors such as tumor hypoxia may play an important role in clinical radioresistance.

Attempts to improve the therapeutic efficacy of radiation include the concomitant administration of chemotherapeutic agents. However, the lack of differential effects on tumors and some normal tissues limits the potential therapeutic efficacy of combining chemotherapeutic agents and radiotherapy due to associated toxicities. One of the most widely used antineoplastic agent, as well as a clinical radiation sensitizer, is 5-FU. In addition to its antitumor effects, 5-FU has been shown in several prospective clinical trials to enhance radiation response and local tumor control in several types of human malignancies (2–6). The enhancement of radiation by 5-FU may involve inhibition of the mechanisms of cell repair as a result of depletion of cellular nucleotides and eradication of radioresistant cells in the S phase of the cell cycle and additive cytotoxic tumor killing effects.

Gene therapy combined with radiotherapy represents a new approach for cancer treatment by selectively radiosensitizing malignant cells. We have previously described the concept of gene therapy targeted by ionizing radiation, whereby a CD encoding tumor necrosis factor-α, is ligated downstream from a radiation-inducible promoter (7–9). In this way, radiation acts as a "molecular switch" to activate transcription of tumor necrosis factor-α, which modifies the cellular response to radiation. Another gene therapy approach with the potential to enhance the radiation killing of malignant cells is VDEPT (10–21). VDEPT incorporates a viral vector system to deliver a gene encoding a prodrug-activating enzyme to malignant cells. Following intracellular expression of the enzyme, a nontoxic prodrug is converted to a toxic compound to sensitize selectively tumor cells to radiation. Normal cells not expressing the therapeutic gene are not affected. Also, because the toxic drug is only produced intratumorally, systemic toxicity is avoided. Although several enzyme/prodrug systems have been investigated for gene therapy (22, 23), VDEPT has concentrated mainly on two enzyme/prodrug combinations for clinical application. The first is the HSVtk/GCV combination. The HSVtk enzyme phosphorylates GCV to GCV triphosphate, which acts as a chain terminator in DNA synthesis, resulting in the death of dividing cells. The second is the CD/5-FC combination. CD converts nontoxic 5-FC to toxic 5-FU. 5-FU inhibits thymidylate synthetase, blocking the methylation reaction of dUMP to TMP. In this manner, it disrupts DNA synthesis and 5-fluorothymidine triphosphate formation, which is incorporated into RNA, resulting in cell deaths in both dividing and nondividing cells. In addition to its antineoplastic effects, 5-FU also sensitize the tumor cells to irradiation. Furthermore, this approach is reported to result in tumor-specific immunity (24–26). The present in vivo experiments were undertaken to investigate the efficacy of virally directed CD/5-FC combination to enhance the tumoricidal action of ionizing radiation in a human cancer xenograft model.

Materials and Methods

Cell Lines. Studies were performed using SQ-20B cells derived from a patient with squamous cell cancer of the larynx after recurrence following primary radiotherapy (27). The SQ-20B cell line forms undifferentiated squa-
mous cell cancer xenografts in nude mice and is relatively radioresistant ($D_{90}$, 239 cGy; Ref. 28) when compared with other tumor cell lines.

**Growth of Human Tumor Xenografts in Vivo.** Xenografts were grown in the hindlimbs of 6–8-week-old athymic female nude mice (Frederick Cancer Research Institute, Frederick, MD) by s.c. inoculation of $5 \times 10^6$ cells suspended in 100 ml of PBS. All animal procedures followed established guidelines for humane treatment of animals. Tumors were permitted to grow for 10–14 days prior to treatment. At day 0, initial tumor volume was determined by direct measurement with calipers and calculated by the formula $(a \times b \times c/2)$, which is derived from the formula for an ellipsoid ($\pi r^3/6$). Tumor volumes were measured twice/week until animal death or for at least 45 days from vector inoculation. Based on the day 0 tumor volume, mice were randomly assigned to treatment groups ($n = 7$ in each group) such that the mean volume of each treatment group was approximately equal. The effect of treatment was studied in small (mean volume, 199 mm$^3$) and large (mean volume, 1069 mm$^3$) xenografts.

**Gene Transfer.** SQ-20B xenografts were injected with the Ad.CMV.CD vector (GenVec, Inc., Rockville, MD) via direct intratumoral injection using a Hamilton syringe. The adenoviral vector (Ad5) is a recombinant replication-deficient type 5 adenovirus with E1 and E3 deletions. It contains the CD gene downstream of the 5' long terminal repeat sequence under the control of a constitutive CMV promoter. Tumors were injected (twice/week for 2 weeks) with a dose of $10^9$ plaque-forming units/injection on days 0, 4, 7, and 11 (Table 1). The replication competent Adenovirus (RCA) for the vector was $1 \times 10^7$.

**Chemicals and Drug Treatment.** The prodrug 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO.). The prodrug was reconstituted in sterile water to a concentration of 250 mg/12.5 ml of 5-FU at a maximum dose of 800 mg/kg body weight (as limited by the drug solubility), injected i.p. daily, 5 days/week for a total of 12 days (Table 1).

**Xenograft Irradiation.** Irradiated mice were immobilized in Lucite chambers, and the entire body was shielded with lead except for the tumor bed. Tumors were irradiated, 5 Gy/day, 4 days/week, beginning at day 3 (Table 1) to a total dose of 50 Gy using a Maxitron generator (1.88 Gy/min). Irradiation was performed within 2 h after i.p. administration of 5-FU.

**Experimental Design.** To study the potential interaction between the vector Ad.CMV.CD, 5-FU, and X-irradiation, SQ-20B xenografts were grown to a mean volume of 1069 mm$^3$. Animals were assigned to one of five treatment groups ($n = 7$ in each group): a combined treatment group receiving Ad.CMV.CD, 5-FU, and 50 Gy; and four control groups receiving no treatment, 50 Gy alone, Ad.CMV.CD alone, or Ad.CMV.CD plus 5-FU. To compare the effect of combined treatment in small tumors, the same experiment was conducted in xenografts with a mean volume of 199 mm$^3$. An additional control group receiving Ad.CMV.CD and 50 Gy was included. The schedule of delivery of the vector, 5-FU, and irradiation, outlined in Table 1, was chosen to simulate clinical radiotherapy. 5-FU administration was not begun until day 3 to allow for stable transfection of tumor cells with the viral vector.

**Statistical Analysis.** Statistical significance was determined by various statistical tests including ANOVA, Kruskal/Wallis test, and Two-Sample Wilcoxon Rank-Sum test. Data are reported as the percentage of original (day 0) tumor volume and graphed as the fractional tumor volume ± SE.

**Results**

No spontaneous tumor regression was observed in untreated control groups in both large and small tumors. The initial tumor size independently affected the rate of tumor growth across different treatment groups. Tumor doubling time was shorter in small tumors and was statistically significant compared to large tumors, starting at day 4 ($P = 0.0001$) until day 36 ($P = 0.0007$).

**Table 1. Schedule of delivery of viral vector (Ad.CMV.CD) at a dose of 10^9 plaque-forming units (PFU)/injection, 5-FU at a dose of 800 mg/kg body weight, and X-irradiation (XRT) at a dose of 5 Gy/day**

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<td>XRT (5 Gy/day)</td>
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In larger tumors with a mean volume of 1096 mm$^3$ (Fig. 1a), radiation alone produced no volumetric regression compared to untreated controls. Animals in the control group and the radiation alone group were sacrificed at day 14 due to large tumor burden. The Ad.CMV.CD vector alone or combined with 5-FU produced volumetric regression to a mean of 37% (day 14) of the original tumor volume. This volumetric regression was followed by tumor regrowth and doubling by day 35. In tumors transfected with Ad.CMV.CD and treated with 5-FC and 50 Gy, significant volumetric regression to 11% was observed at day 21 ($P = 0.01$). This regression was statistically significant beginning at day 11 ($P = 0.0004$) and continued through day 28 ($P = 0.049$). No cures were observed in these tumors receiving the combined treatment.

In smaller tumors with a mean volume of 199 mm$^3$ (Fig. 1b), neither radiation alone nor Ad.CMV.CD with or without radiation produced volumetric regression below the original tumor volume. Furthermore, no significant tumor control was observed in tumors treated with Ad.CMV.CD and 5-FU injection. Small tumors transfected with Ad.CMV.CD and treated with 5-FC and 50 Gy demonstrated significant volumetric regression to 40% of original tumor volume at day 33 ($P = 0.0005$), with no regrowth. This volumetric regression was significant beginning at day 12 ($P = 0.014$) and continued through day 36 ($P = 0.01$). Three of seven tumors in that combined group were cured at day 26 with no recurrence.

No treatment-related deaths occurred in treatment groups transfected with the vector and treated with 5-FC and radiation. Mild local edema and skin desquamation were observed in treatment groups receiving radiation therapy.

**Discussion**

Previous experiments using VDEPT has shown that the CD/5-FU system was not effective when xenograft volume was large. The present experiment was designed to study if the CD/5-FC combination interacts with irradiation to improve tumor control in large tumors. This study clearly demonstrates that 5-FC selectively enhances the tumoricidal action of ionizing radiation in xenografts expressing the CD gene.

The interaction between 5-FU and irradiation has been experimentally studied in a variety of dosages, schedules, and modes of administration without any clear demonstration of therapeutic advantage of one particular regimen (29–35). In this experiment, we administered the drug daily concomitant with radiation therapy to simulate clinical protocols. The i.p. route was chosen based on pharmacokinetic studies of 5-FU in nude mice (13). Previous studies have shown that 5-FU is not toxic to nude mice or other mouse strains (11, 24, 36). By contrast, 5-FU was relatively toxic to nude mice when given via i.p. or oral route at a dose of 25 and 32 mg/kg, respectively. The half-life of 5-FU is approximately 40 min in nude mice, and plasma levels of 25 mg or greater could be maintained for approximately 4 h following a single i.p. injection of 500 mg of 5-FU/kg of body weight (13). Huber et al. (13) also demonstrated that the i.p. route was more efficacious than continuous tail infusion. Pharmacokinetics studies (14, 37) of 5-FU demonstrated that intratumoral 5-FU reached a maximal level at approximately 45 min after 5-FU administration, with peak levels seven times greater than those following systemic 5-FU administration. Furthermore, 5-FU levels remained elevated for a longer time, and there was a 17-fold increase in the area under the curve of intratumoral 5-FU compared to systemic drug administration.

The efficacy of VDEPT is dependent not only on the efficiency of gene transfer but also upon the enzyme/prodrug system. The expression of CD enzyme in colon tumor cells has resulted in a 600-fold increase in enzyme activity compared to normal colon tissues.
increase in sensitivity to the prodrug 5-FC (13). Huber et al. (14) have also reported that when only 2% of colorectal tumor mass contains CD-expressing cells, significant regression in all tumors was observed when mice were treated with nontoxic levels of 5-FC. Similar results were reported by Trinh et al. (38). This bystander effect due to the readily diffusible toxic catabolite 5-FU (14, 39) appears to overcome low gene transfer efficiency and accounts for the significant therapeutic effect. In the present experiment, a significant bystander therapeutic effect is evident by the tumor regression observed. A similar bystander effect has also been described with the HSVtk/GCV system, both in culture and in vivo (40–53). No cell contact is needed with the CD/5-FC system, as opposed to the HSVtk/GCV combination, which requires metabolic cooperation between cells through gap junctions (40, 43–45) and/or transfer of apoptotic vesicles (46, 47). The in vivo mechanism of this bystander effect may require an intact immunological system (50–53). Trinh et al. (38) has shown recently that the CD/5-FC combination is more efficacious compared to HSVtk/GCV in controlling WiDr colorectal xenografts when only a small percentage of the tumor cells expressed the therapeutic gene due to a greater bystander effect.

Khil et al. (54) have reported that tumor cells transfected with the CD gene are sensitized to radiation killing in vitro when exposed to 5-FC for 72 h prior to radiation. This radiation enhancement effect was evident only in preirradiated cells and was dependent on the drug concentration and preirradiation exposure time. Similar radiation enhancement effects have been reported with various enzyme/pro-drug combinations other than CD/5-FC. Recent reports have demonstrated that the HSVtk/bromovinyl deoxyuridine enzyme/prodrug combination enhances radiation cytotoxicity in human glioma cells (19, 55–57). U-251 human glioma cells transduced with HSVtk and exposed to 40 μg/ml of bromovinyl deoxyuridine for 24 h prior to irradiation were more sensitive to radiation compared to control cells. Kim et al. (55) reported recently that the antiviral agents bromovinyl deoxyuridine and acyclovir, administered systemically, selectively enhanced...
radiation cytotoxicity in rat 9L gliosarcoma tumor cells transfected with the HSVtk gene both in vitro and in vivo.

The CD5-FC enzyme/prodrug system is of particular clinical interest because the enzyme CD is not found in mammalian cells, and the prodrug 5-FC is relatively nontoxic in humans (58, 59). 5-FU can readily diffuse into and out of cells, crossing biological membranes by nonfacilitated diffusion, accounting for a significant therapeutic bystander effect, which overcomes the need for targeted gene expression in every tumor cell. Through its mechanisms of action involving DNA and RNA pathways, 5-FU results in deaths of both dividing and nondividing cells. 5-FU is also the radiosensitizing agent of choice presently used in the treatment of several human tumors.

The finding that no significant volumetric regression occurred in tumors treated with Ad.CMV.CD and 5-FC is explained by our previous finding that SQ-20B tumors are not sensitive to 5-FU (28). An unexpected and interesting finding was the observation that large tumors injected with Ad.CMV.CD grew more slowly than un.injected control tumors. The effect of the vector alone on tumor growth could be due to a direct cytotoxic effect of the adenovirus or to an immunological effect as a result of: more effective antigen presentation from dead CD+ cells; inflammatory response to CD+ dying cells with recruitment and activation of antigen presenting cells; or immunogenicity of the CD protein itself. A direct viral cytotoxic effect seems unlikely because we have demonstrated previously that treatment with adenoviral vector with or without radiation produced no significant tumor control in SQ-20B xenografts (8). This finding led us to add an additional control group, receiving Ad.CMV.CD plus irradiation in the second experiment with smaller tumors, to determine whether the presence of the Ad.CMV.CD plays a role in tumor regression when combined with radiation. No tumor regression below the original tumor volume was observed in this group, confirming our previous finding (8). Taken collectively, these data suggest that CD5-FC combination is effective as a radiosensitization strategy in vivo, especially in tumors that cannot be controlled effectively with radiation alone or tumors not sensitive to 5-FU.

Conclusion. VDEPT combined with radiotherapy for the treatment of cancer offers a new potential to improve the therapeutic efficacy of radiation through selective tumor cell radiosensitization. Radiosensitization via local conversion of 5-FC to 5-FU can potentially lead to more tolerable treatment without the local or systemic toxicity of conventionally delivered 5-FU. This approach would be particularly useful for the treatment of large unresectable tumors or tumors with a high risk failure due to further regression. Additional studies are needed to determine the optimal radiosensitizing dose, as well as the duration and sequence of delivery of 5-FC in relation to radiation therapy.

References

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