

# External Beam Radiation of Tumors Alters Phenotype of Tumor Cells to Render Them Susceptible to Vaccine-Mediated T-Cell Killing

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## ABSTRACT

Local radiation is an established therapy for human tumors. Radiation also has been shown to alter the phenotype of target tissue, including gene products that may make tumor cells more susceptible to T-cell-mediated immune attack. We demonstrate a biological synergy between local radiation of tumor and active vaccine therapy. The model used consisted of mice transgenic for human carcinoembryonic antigen (CEA) and a murine carcinoma cell line transfected with CEA. The vaccine regimen consisted of a prime and boost strategy using vaccinia and avipox recombinants expressing CEA and three T-cell costimulatory molecules. One dose of 8-Gy radiation to tumor induced up-regulation of the death receptor Fas *in situ* for up to 11 days. However, neither radiation at this dose nor vaccine therapy was capable of inhibiting growth of 8-day established tumor. When vaccine therapy and local radiation of tumor were used in combination, dramatic and significant cures were achieved. This was mediated by the engagement of the Fas/Fas ligand pathway because Ag-bearing tumor cells expressing dominant-negative Fas were not susceptible to this combination therapy. Following the combination of vaccine and local radiation, tumors demonstrated a massive infiltration of T cells not seen with either modality alone. Mice cured of tumors demonstrated CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses specific for CEA but also revealed the induction of high levels of T-cell responses to two other antigens (gp70 and p53) overexpressed in tumor, indicating the presence of a consequential antigen cascade. Thus, these studies demonstrate a new paradigm for the use of local tumor irradiation in combination with active specific vaccine therapy to elicit durable antitumor responses of established tumors.

## INTRODUCTION

Colorectal carcinoma has long been considered poorly immunogenic and thus less responsive to active immunotherapy. CD8<sup>+</sup> CTLs have been described to play crucial roles in host defense against malignancies in mouse models and humans (1). However, tumors grow progressively in the host despite their antigenicity and the presence of potentially tumor-reactive lymphocytes (2, 3). Thus, we have been interested in combining other modalities of cancer treatment along with active immunotherapy to potentially improve the T cell-tumor cell interaction, which may have implications for designing improved therapeutic strategies. One combination involves localized, external beam irradiation of solid tumors and active vaccination with recombinant tumor-associated antigen (TAA)-based vaccines.

The conventional use of radiation as an anticancer modality has been to exploit its cytotoxic properties. In tumor and normal tissue, radiation causes an inflammatory response that includes cytokine release, up-regulation of adhesion molecules, and a subsequent increase in lymphocyte adhesion (4). However, recent *in vitro* studies also have suggested that sublethal doses of irradiation can induce

immune modulatory effects, such as up-regulation of cell surface expression of MHC determinants, costimulatory molecules, or Fas (CD95), on hematologic and nonhematologic cells (3–5). Fas, a member of the tumor necrosis factor (TNF) receptor family, is a death receptor that induces apoptosis on ligation with agonist anti-Fas antibody or the natural Fas ligand (FasL). Fas-mediated apoptosis plays important roles in the immune system, including the apoptotic selection process during T-cell development, clonal deletion of autoreactive T cells in the periphery, and as an effector mechanism of CTL (6, 7). In mouse models, it has been demonstrated that CD8<sup>+</sup> CTLs mediate lysis of susceptible targets via two major effector mechanisms following MHC/Ag recognition: (a) secretion of perforin and granzyme contents, and (b) engagement of Fas by FasL expressed by the activated CTL (8, 9). Both pathways lead to apoptotic cell death involving caspase-dependent and/or -independent signaling events (8). To evaluate the potential role of the Fas/FasL pathway in the endogenous host response against solid tumors and the potential therapeutic utility of manipulating Fas expression on tumor cells, we examined the regulation of Fas expression by relatively low doses of local radiation of tumors in combination with recombinant anticancer vaccines.

Active specific immunotherapy with a TAA-specific vaccine regimen was used to induce and potentiate T-cell responses against carcinoembryonic antigen (CEA) in combination with local irradiation of s.c. tumors. CEA expression is prevalent among diverse human carcinomas, namely, colorectal, gastric, pancreatic, breast, and non-small cell lung malignancies (10). Transgenic mice expressing the human CEA gene as “self” in tissues similar to expression in humans were used as a more relevant preclinical model to investigate various experimental immunotherapies (11). We sought to examine the potential synergy of a poxvirus-based CEA vaccine regimen in combination with external-beam irradiation of local s.c. CEA-expressing tumors. CEA-Tg mice were vaccinated with a poxvirus-based diversified prime and boost regimen: recombinant vaccinia prime (rV) followed by recombinant fowlpox boosts (rF). These recombinant viruses encoded the CEA gene and genes for a triad of costimulatory molecules [TRICOM; B7-1, intercellular adhesion molecule 1 (ICAM-1), and leukocyte function associated antigen 3]. The advantages of the diversified vaccine prime and boost regimen have been described (12–14), as has the use of T-cell costimulation via inserting multiple costimulatory molecules into these vectors (15–17). The rV-CEA/TRICOM/rF-CEA/TRICOM vaccine regimen was used in combination with sublethal irradiation of the tumor.

We have shown previously that sublethal doses of external-beam radiation to murine MC38-CEA<sup>+</sup> adenocarcinoma cells *in vitro* made them more sensitive to CEA-specific CTL-mediated lysis (18). The radiation was shown to up-regulate Fas on the tumor cells, and the enhanced CTL sensitivity was blocked completely by anti-Fas antibody. Irradiation of an 8-day s.c. tumor (8 Gy) in combination with the adoptive transfer of CEA-specific CTL resulted in significant antitumor effects, whereas radiation alone or CTL adoptive transfer alone had no effect on tumor growth. In the results reported here, we have used a CEA-transgenic mouse model and a CEA-based vaccine

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regimen to examine (a) the effect of sublethal doses of local tumor irradiation on Fas expression *in vitro* and *in vivo*; (b) the duration of this up-regulation after exposure of tumor cells to radiation; (c) enhanced susceptibility of tumor cells to vaccine-induced CTL-mediated killing; and (d) the role of Fas/FasL interactions in tumor cell destruction *in vitro* and *in vivo*.

Overall, these results revealed for the first time that the regulation of Fas expression by tumor cells via sublethal irradiation significantly improved the therapeutic efficacy of a recombinant anticancer vaccine regimen. Furthermore, localized irradiation of s.c. tumors in combination with vaccine led to a dramatic influx of CD8<sup>+</sup> cytotoxic T cells to the tumor microenvironment and subsequent inhibition of tumor growth. In addition, we demonstrated the induction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells specific for multiple TAA not encoded by the vaccine was observed after the combination therapy. Moreover, up-regulated Fas on the tumors was shown to be solely responsible for the enhancement of the efficacy of the vaccine therapy in this model system, as determined by the inability of the combination therapy to mediate tumor regression of tumor cells defective in Fas signaling. Thus, irradiation of tumor cells may induce unique immunoregulatory properties that facilitate antitumor activity by engaging the lytic capacity of Ag-specific CTL, which may have important implications for the combination of immunotherapy and radiation therapy.

## MATERIALS AND METHODS

**Tumor Cells.** The murine colon adenocarcinoma cell line MC38 (H-2<sup>b</sup>) has been described previously (19). MC38 cells expressing human CEA were generated by retroviral transduction with CEA cDNA and are designated MC38-CEA<sup>+</sup>. MC38-CEA<sup>+</sup> cells were transfected with an expression vector encoding dominant-negative (DN-1) Fas (Ref. 20; a gift from Dr. Robert H. Wiltrout, NCI, NIH, Frederick, MD). These cells are designated MC38-CEA-DN-1.

**Animals.** For *in vivo* studies, 6–8-week-old female C57BL/6 or CEA-Tg mice were used. The generation and characterization of the CEA-Tg mouse have been described previously (11). Mice were housed and maintained under pathogen-free conditions in microisolator cages.

**Tumor Irradiation.** The MC38, MC38-CEA<sup>+</sup>, and MC38-CEA-DN-1 cells were harvested while in log-growth phase. For *in vitro* studies, tumor cells were placed on ice and irradiated (8 Gy) as described previously (18). For *in vivo* studies, mice were injected with  $3 \times 10^5$  tumor cells s.c. in the right hind leg. Tumors were measured by digital caliper, and at day 14, when the tumors reached a mean volume of 75–100 mm<sup>3</sup>, irradiation (2–30 Gy) was initiated as described previously (18).

**Tumor Cell Characterization.** After irradiation, tumors were removed surgically at several time points and (a) fixed, sectioned at 5  $\mu$ m, and stained with anti-Fas monoclonal antibody (mAb; clone M-20; Santa Cruz Biotechnology, Santa Cruz, CA) or an isotype-matched control antibody for immunohistochemical analysis; (b) frozen, sectioned at 5  $\mu$ m, and stained with anti-CD3 mAb for immunohistochemical analysis; or (c) made into single-cell suspension for flow cytometric analysis.

Cell-surface staining was performed with primary FITC-labeled mAb COL-1 (anti-CEA), H-2K<sup>b</sup>, and ICAM-1 (CD54; Ref. 21). Fas cell-surface staining was performed with primary PE-labeled mAb. For analysis of tumor-infiltrating cells, tumor cell suspensions were stained with primary labeled antibody specific for CD3, CD4, CD8, CD19, and natural killer cells. All of the mAbs were purchased from PharMingen (San Diego, CA). Immunofluorescence was analyzed and compared with the appropriate isotype-matched controls (PharMingen) with a FACScan cytometer using Cellquest software (Becton-Dickinson, Mountain View, CA). Nonviable cells were excluded electronically from analysis based on propidium iodide exclusion.

**Cytotoxicity Assays.** The H-2D<sup>b</sup>-restricted, CEA-specific CD8<sup>+</sup> CTL line was generated from CEA-Tg mice (C57BL/6 background, H-2<sup>b</sup>) as described and recognizes the peptide epitope CEA<sub>526–533</sub> (EAQNTTYL; Ref. 22). MC38-CEA<sup>+</sup> tumor cells were prepared for use as targets in a standard cytotoxicity assay using <sup>51</sup>Cr as described previously (23). These radiolabeled

cells ( $5 \times 10^3$  cells/well) were incubated with 1  $\mu$ g/ml CEA peptide or VSVN peptide (vesicular stomatitis virus, RGYVYQGL) as a negative control and cocultured with CEA-specific CTL at various effector:target ratios for 4 h at 37°C with 5% CO<sub>2</sub> (24). MC38-CEA<sup>+</sup> or MC38-CEA-DN-1 tumor cells were mock irradiated (0 Gy) or irradiated (8 Gy), recultured for 24 h, and then prepared for use as targets using <sup>51</sup>Cr. CEA-specific CTL and targets ( $5 \times 10^3$  cells/well) were combined at effector:target ratios ranging from 50:1 to 6.25:1 in 96-well U-bottomed plates (Costar, Cambridge, MA) and incubated for 18 h at 37°C with 5% CO<sub>2</sub>. For indicated experiments, the CEA-specific CTLs were preincubated for 2 h in the presence of 100 nM concanamycin A (CMA; to specifically inhibit perforin-dependent lysis) and incubated with target cells with CMA present during the assay. After incubation, supernatants were collected, and the percentage of specific release of <sup>51</sup>Cr was determined as described previously (18).

**Functional Fas Assay.** MC38-CEA<sup>+</sup> or MC38-CEA-DN-1 tumor cells were nonirradiated (0 Gy) or irradiated (8 Gy) and recultured for 24 h. Cells then were labeled with <sup>51</sup>Cr as described previously and analyzed for Fas-mediated killing by incubation for 18–24 h with anti-Fas mAb (Jo2; PharMingen) plus protein G (10  $\mu$ g/ml; Amersham Pharmacia, Piscataway, NJ). Control wells consisted of tumor cells incubated with isotype-matched antibody.

**Recombinant Poxvirus Vaccines.** The recombinant vaccinia and fowlpox viruses containing the human CEA gene and the murine B7-1, ICAM-1, and leukocyte function associated antigen 3 genes (designated rV-CEA/TRICOM and rF-CEA/TRICOM, respectively) have been described previously (17). The recombinant fowlpox virus containing the gene for murine granulocyte macrophage colony-stimulating factor (GM-CSF) has been described previously (25). Drs. A. Gomez-Yafal, D. Panicali, G. Mazzara, and L. Gritz of Therion Biologics Corporation (Cambridge, MA) provided the Orthopox viruses as part of an ongoing collaborative research and development agreement between the NCI/NIH and Therion Biologics Corporation.

**Tumor Therapy Studies.** MC38, MC38-CEA<sup>+</sup>, or MC38-CEA-DN-1 cells ( $3 \times 10^5$ ) were injected s.c. in the quadriceps area of the right hind limb. Eight days following tumor transplant, mice were vaccinated s.c. once with  $1 \times 10^8$  plaque-forming units rV-CEA/TRICOM admixed with  $1 \times 10^7$  plaque-forming units rF-GM-CSF. Tumors were irradiated with 8 Gy on day 14 following tumor transplant or with 2 Gy/day from day 11 to day 14. On day 15, mice were boosted with  $1 \times 10^8$  plaque-forming units rF-CEA/TRICOM admixed with  $1 \times 10^7$  plaque-forming units rF-GM-CSF. This booster vaccination regimen was repeated two additional times at 7-day intervals. Tumors were measured daily by digital caliper in two dimensions, and the volumes were calculated as described previously (13). Animals were sacrificed when any tumor measurement (length or width) was >20 mm.

**Immunologic Assays.** CEA-specific CD4<sup>+</sup> T-cell responses of mice were analyzed as described previously (13). The p53-specific CD4<sup>+</sup> T-cell responses were analyzed in a similar manner using an MHC-II p53<sub>108–122</sub> peptide (0.16–2.5  $\mu$ g/ml, LGFLQSGTAKSVMT; Ref. 26). To evaluate CD8<sup>+</sup> T-cell responses, spleens were removed as indicated, dispersed into single-cell suspensions, pooled, and cocultured with the CEA peptide (10  $\mu$ g/ml), the H-2D<sup>b</sup>-restricted peptide p53<sub>232–240</sub> (2  $\mu$ g/ml, KYMCNSSCM; Refs. 23, 27), or the H-2K<sup>b</sup>-restricted peptide p15E<sub>604–611</sub> (1  $\mu$ g/ml, KSPW-FTTL, referred as gp70 peptide; Ref. 28) for 7 days. Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient (Beckman Coulter, Fullerton, CA). T cells were restimulated with fresh irradiated naive splenocytes and the corresponding peptide for 24 h. As control peptides, VSVN was used for H-2D<sup>b</sup>-restricted peptides, or ovalbumin<sub>257–264</sub> (SIIN-FEKL) was used for H-2K<sup>b</sup>-restricted peptides (29). Supernatant was collected and analyzed for murine IFN- $\gamma$  by cytometric bead array (PharMingen) according to the manufacturer's instructions.

**Autoimmune Assays.** Mice that were successfully treated by the combination of vaccine therapy and external-beam radiation were monitored for 6 months. Control mice consisted of age-matched CEA-Tg mice that did not receive tumor and were not vaccinated. On sacrifice, serum was analyzed for autoantibodies. Antibodies to antinuclear antibody (ANA), rheumatoid factor, nuclear ribonuclear protein, histone, SCL-70 (DNA topoisomerase I), dsDNA, ssDNA, and circulating immune complexes were determined in a qualitative or semiquantitative manner (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions.

**Statistical Analysis of the Data.** When indicated, the results of tests of significance are reported as *P*s and are derived from Student's *t* test using a two-tailed distribution. *P*s were calculated at 95% using Statview 4.1 (Abacus Concepts Inc., Berkeley, CA) software package.

## RESULTS

**Expression and Persistence of Up-Regulated Fas on Tumor Cells after Irradiation.** Because Fas has been shown to be up-regulated on tumor cells *in vitro* after irradiation, we sought to determine the duration of Fas up-regulation *in vivo* following radiation by monitoring its expression over time (18). C57BL/6 mice were injected with MC38-CEA<sup>+</sup> tumor cells s.c. After 14 days, the tumors were irradiated (8 Gy), and another subset of mice was mock irradiated. The tumors were excised starting from 2 days after radiation up to 11 days of irradiation and examined for Fas up-regulation by immunohistochemical staining and flow cytometric analysis (Fig. 1). In these studies, CEA-positive cells were gated to confirm them as tumor cells. Nonirradiated MC38-CEA<sup>+</sup> tumor cells exhibited a low level (5%) of Fas expression (Fig. 1A). Following irradiation, up-

regulated Fas was noted after 2 days (Fig. 1B). The number of Fas-positive tumor cells reached its peak at day 7 after radiation (89%; Fig. 1D), which was maintained up to 11 days (Fig. 1E). These observations were confirmed by immunohistochemistry (Fig. 1, F–J). Histopathologic analysis revealed there was no significant contribution of Fas-expressing leukocytes as shown by H&E staining (Fig. 1, P–T).

To further characterize the phenotypic changes induced by radiation, we also examined cell surface expression of Fas, ICAM-1, and MHC class I molecules on s.c. implanted MC38-CEA<sup>+</sup> tumors following several dose levels (2, 8, 10, or 30 Gy) of irradiation. Irradiation of MC38-CEA<sup>+</sup> tumors induced strong expression of Fas in a dose-dependent manner, with the greatest up-regulation of Fas noted at 8 and 10 Gy at 7 days after radiation (data not shown). Irradiation of tumor with the 30-Gy dose rapidly increased the level of Fas (98% of cells within 2 days); however, this dose level caused substantial toxicity, and later time points were not analyzed. Irradiation also increased the expression of ICAM-1 on tumor cells in a dose-dependent manner. Following 8 Gy and 10 Gy there was a marked increase

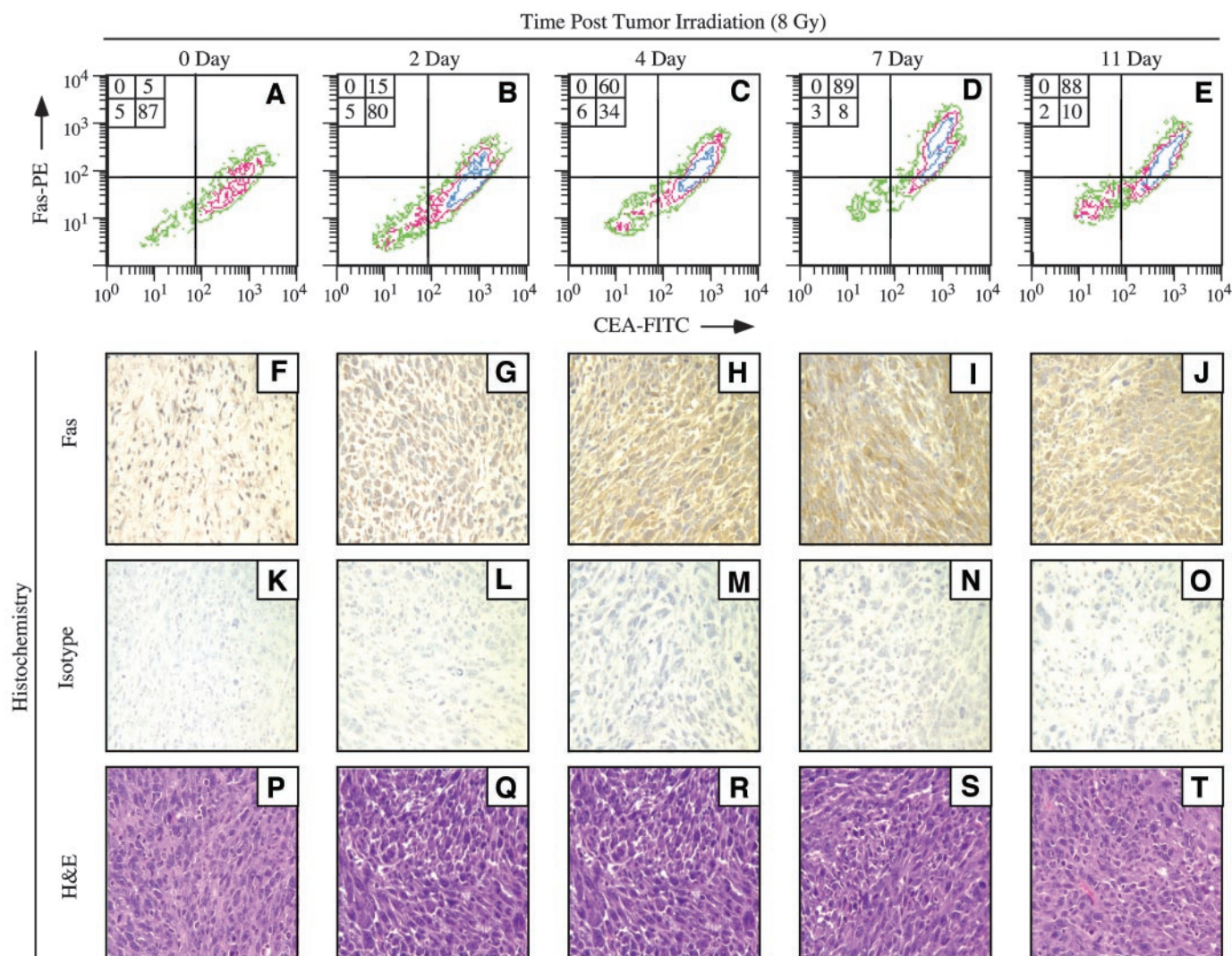


Fig. 1. Expression of up-regulated Fas on tumor cells after irradiation is maintained for >11 days. C57BL/6 mice were injected with  $3 \times 10^5$  MC38-CEA<sup>+</sup> tumor cells s.c. Fourteen days later, tumors were subjected to external-beam irradiation (8 Gy). Tumors were removed surgically at several time points after radiation and analyzed for Fas expression by flow cytometry and immunohistochemistry. Tumors were excised 0 (A), 2 (B), 4 (C), 7 (D), or 11 (E) days after radiation and costained with carcinoembryonic antigen (CEA) and Fas antibodies. Insets, percentage of positive cells for each quadrant. To confirm Fas expression, tumors were harvested 0 (F), 2 (G), 4 (H), 7 (I), or 11 (J) days after radiation and immunostained with anti-Fas monoclonal antibody (F–J) or an isotype control antibody (K–O). Also shown are the corresponding H&E-stained sections (P–T). Photomicrographs are shown at 40 $\times$ .

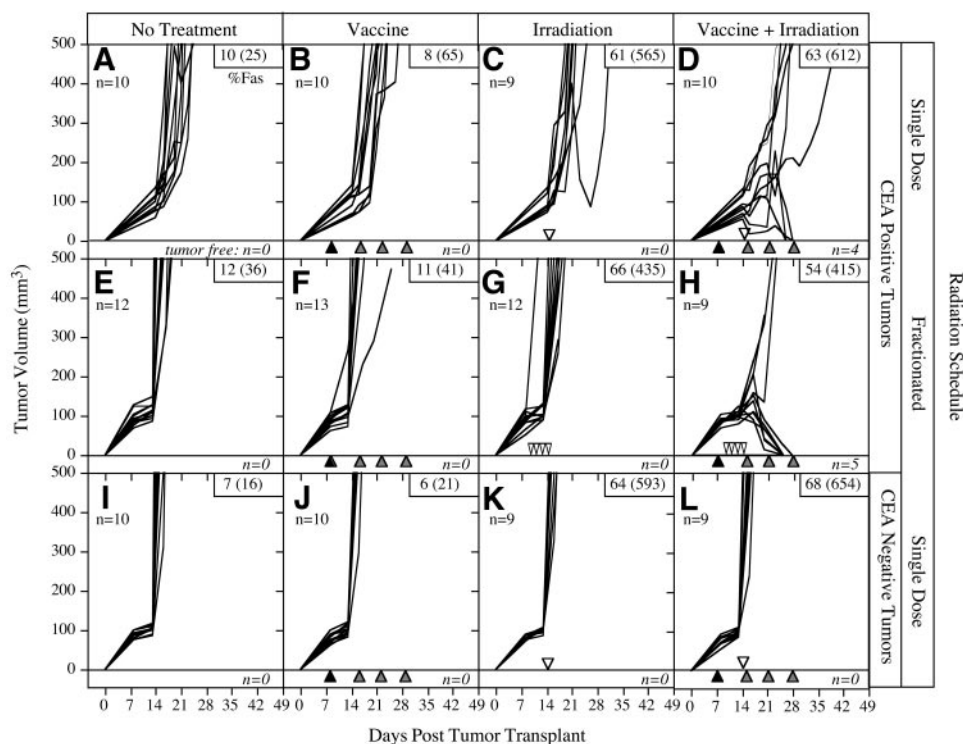


Fig. 2. Irradiation of tumor cells *in vivo* enhances efficacy of vaccine therapy. Carcinoembryonic antigen (CEA)-Tg mice were injected with MC38-CEA<sup>+</sup> tumor cells s.c. *A*, mice received no treatment. *B*, mice were vaccinated with recombinant vaccinia (rV)-CEA/triad of costimulatory molecules (TRICOM) on day 8 (▲), followed by boosting with recombinant fowlpox (rF)-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *C*, tumors in mice were subjected to single-dose external-beam irradiation (8 Gy) *in situ* on day 14 (▽). *D*, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *E*, mice received no treatment. *F*, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *G*, tumors in mice were subjected to fractionated external-beam irradiation (2 Gy) *in situ* on days 11, 12, 13, and 14 (▽). *H*, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲). Tumors were subjected to fractionated external-beam irradiation (2 Gy) *in situ* on days 11, 12, 13, and 14 (▽), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *I*, mice received no treatment. *J*, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *K*, tumors in mice were subjected to single-dose external-beam irradiation (8 Gy) *in situ* on day 14 (▽), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). *L*, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲). Tumors were subjected to single-dose external-beam irradiation (8 Gy) *in situ* on day 14 (▽), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). In a subset of mice from each treatment group, tumors were removed surgically at day 21 post-tumor transplant and costained with CEA and Fas antibodies. *Inset*, percentage of Fas-positive cells (mean fluorescent intensity). All of the vaccines were coadministered with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored.

in the cell surface density of MHC class I (H2-K<sup>b</sup>). For the subsequent studies, mice were irradiated with 8 Gy.

**Irradiation of Tumor Cells *in Vivo* Enhances Efficacy of Vaccine Therapy.** To examine whether sublethal irradiation of growing tumors improves tumor rejection by a recombinant anticancer vaccine regimen, we vaccinated mice with a diversified prime and boost regimen with CEA/TRICOM vectors in combination with local radiation of the tumor. The vaccine regimen consisted of priming mice with rV-CEA/TRICOM admixed with rF-GM-CSF followed by three weekly boosts with rF-CEA/TRICOM admixed with rF-GM-CSF. MC38-CEA<sup>+</sup> tumor cells were injected s.c. in the right hind leg of CEA-Tg mice. Eight days following tumor transplant, groups of mice then were divided into those that received (a) no treatment; (b) vaccine alone; (c) irradiation of the tumor alone; or (d) the combination of vaccination followed by radiation (Fig. 2). Tumors of mice that did not receive any treatment grew progressively, ultimately causing the death of the animals (100% by day 30; Fig. 2A). Therapy of tumors with rV-CEA/TRICOM priming on day 8 followed by rF-CEA/TRICOM boosting on days 15, 22, and 29 (Fig. 2B) did not significantly inhibit tumor growth ( $P = 0.849$  as compared with no treatment). Irradiation of tumors (8 Gy) on day 14 (Fig. 2C) also failed to significantly impact the extent of tumor growth in these mice ( $P = 0.282$  as compared with no treatment). However, therapy of tumors with the combination of the vaccine regimen and irradiation resulted in a marked and significant decrease in tumor growth rate and tumor volume ( $P = 0.007$  versus no treatment;  $P = 0.001$  versus

irradiation alone;  $P = 0.001$  versus vaccine alone; Fig. 2D). In addition, 40% of the mice treated with the combination of irradiation and vaccine therapy resolved their tumor mass and remained tumor free for the duration of the experiment (180 days). To extend the observation that combination therapy of TAA-specific vaccination in conjunction with local radiation enhances the efficacy of vaccine therapy, we tested the treatment schedule in a TAA-negative tumor model with parental MC38 tumor cells (CEA negative; Fig. 2, I–L). Mice treated with the CEA/TRICOM vaccine regimen in combination with 8 Gy local tumor radiation (Fig. 2L) did not show any significant delay in tumor growth compared with the mice that were left untreated (Fig. 2I), treated with the vaccine regimen (Fig. 2J), or treated with irradiation (Fig. 2K), confirming the role of TAA-specific immune responses in the tumor therapy study of MC38-CEA<sup>+</sup> cells.

In cancer patients, fractionation of radiation dose is a common clinical practice to reduce radiation-induced toxicity and to enhance antitumor activity. To validate the utility of dose fractionation in combination with vaccine therapy, the aforementioned tumor therapy was repeated using the fractionated radiation dose of 2 Gy/day for 4 days (days 11–14; Fig. 2, G and H). Mock-irradiated MC38-CEA<sup>+</sup> tumor cells again demonstrated low levels of Fas (12%; Fig. 2E, *inset*). Following fractionated radiation, these cells demonstrated significant Fas up-regulation (66%; Fig. 2G, *inset*) that was comparable with that seen after 8 Gy administered as a single dose (Fig. 2C, *inset*). Again, in this study, only the combination of the CEA/TRICOM vaccine regimen and fractionated irradiation resulted in a significant

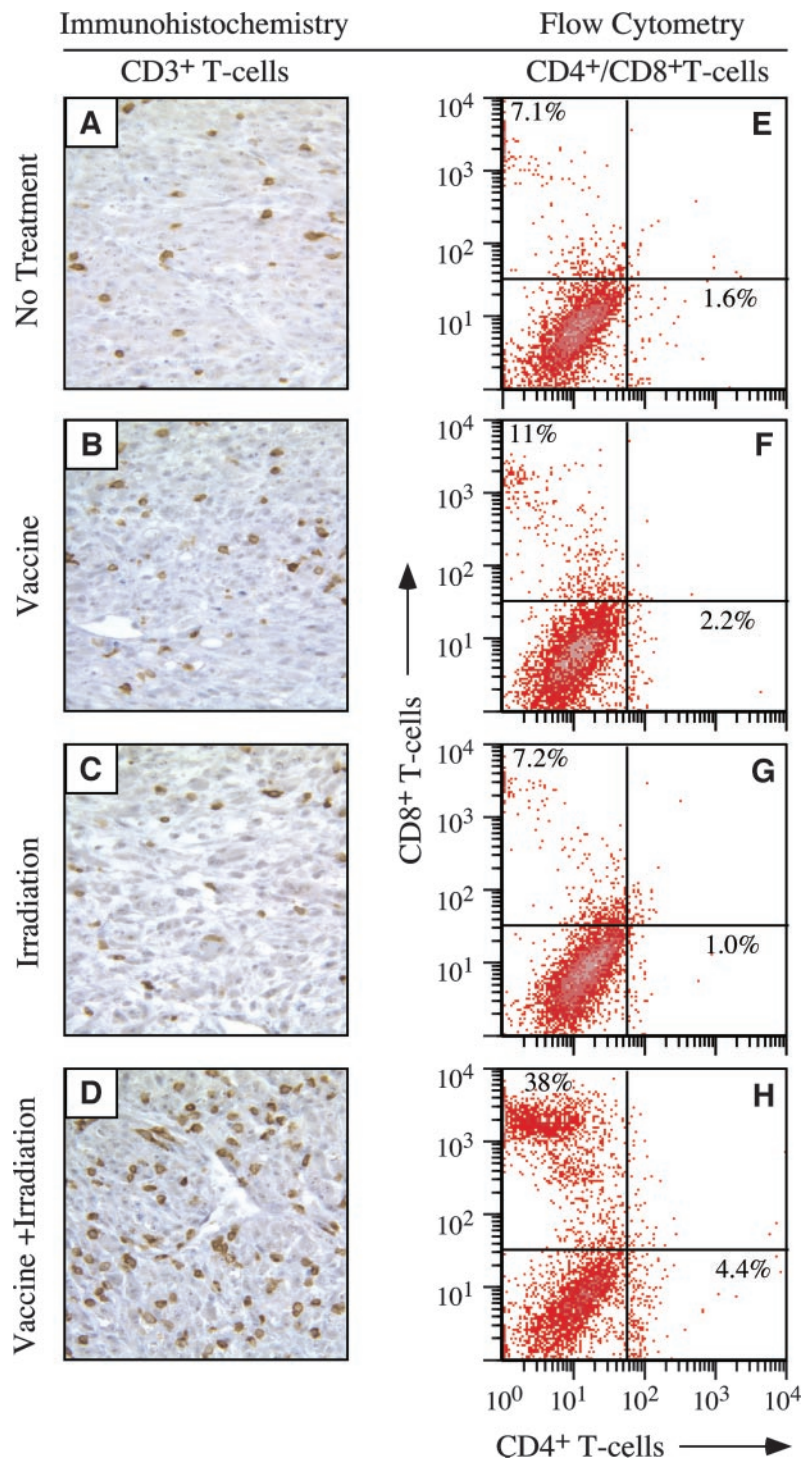


Fig. 3. Characterization of infiltrating cells of tumors treated with the combination of vaccine therapy and radiation. Carcinoembryonic antigen (CEA)-Tg mice were injected with MC38-CEA<sup>+</sup> tumor cells s.c. *A* and *E*, mice received no treatment. *B* and *F*, mice were vaccinated with recombinant vaccinia (rV)-CEA/triad of costimulatory molecules (TRICOM) on day 8, boosted with recombinant fowlpox (rF)-CEA/TRICOM on day 15. *C* and *G*, tumors in mice were subjected to irradiation (8 Gy) *in situ* on day 14. *D* and *H*, mice were vaccinated with rV-CEA/TRICOM on day 8. Tumors were irradiated (8 Gy) *in situ* on day 14, followed by boosting with rF-CEA/TRICOM on day 15. All of the vaccines were coadministered with rF-granulocyte macrophage colony-stimulating factor. Tumors were removed surgically at day 21 after radiation and analyzed for infiltrating CD3<sup>+</sup> T cells by immunohistochemistry (*A–D*) or CD4<sup>+</sup> and CD8<sup>+</sup> T cells by flow cytometry (*E–H*).

reduction of tumor burden ( $P = 0.001$  versus untreated mice;  $P = 0.002$  versus radiation alone). In addition, 55% of these mice resolved their tumor mass and remained tumor free for the duration of the experiment (180 days). There was no significant difference in the therapeutic efficiency of the vaccine regimen in combination with single dose or fractionated radiation ( $P = 0.3$ ). Two additional radiation schedules were tested in combination with the CEA/TRICOM vaccine regimen: 2 Gy once per week for 4 weeks or 8 Gy once a week for 4 weeks. These schedules did not work as efficiently as the schedule shown in Fig. 2 (data not shown). These experiments were repeated four times with similar results.

**Characterization of Infiltrating Cells of Tumors Treated with the Combination of Vaccine Therapy and External-Beam Radiation.** To evaluate the role of immune cells in the significant reduction of tumor growth after combination of vaccine therapy and radiation, we determined whether infiltrating cells were present in the tumor microenvironment following treatment. MC38-CEA<sup>+</sup> cells were injected s.c. in the right hind leg of CEA-Tg mice. Eight days later, groups of mice then were divided into those that received (*a*) no treatment; (*b*) vaccine alone; (*c*) irradiation of the tumor alone; or (*d*) the combination of vaccination followed by radiation. Tumors were surgically removed 7 days after radiation and analyzed for infiltrating

Table 1 Cellular immune responses to multiple tumor antigens after therapy with vaccine and radiation

Treatment <sup>d</sup>	Antigen <sup>a</sup>										
	ConA	HSA	CEA ( $\mu\text{g/ml}$ ) <sup>b</sup>			p53 ( $\mu\text{g/ml}$ )			Peptide <sup>c</sup>		
			25	12.5	6.25	1.25	0.625	0.3125	CEA	p53	gp70
Vaccine + Radiation	930	0.8	3.9	2.9	1.7	3.8	3.6	2.6	2,000	320	28,940
Control	887	1.3	1.1	0.8	1.0	1.2	0.7	0.7	<2.5 <sup>e</sup>	<2.5	<2.5

<sup>a</sup> For proliferation, antigen concentrations were ConA (2.5  $\mu\text{g/ml}$ ), human serum albumin (25  $\mu\text{g/ml}$ ), CEA (25–6.25  $\mu\text{g/ml}$ ), or p53 (1.25–0.3125  $\mu\text{g/ml}$ ). Each value represents the stimulation index of the mean CPM of triplicate samples *versus* media. SD never exceeded 10%.

<sup>b</sup> CEA, carcinoembryonic antigen; ConA, concanavalin A; rV, recombinant vaccinia; TRICOM, triad of costimulatory molecules; rF, recombinant fowlpox; GM-CSF, granulocyte macrophage colony-stimulated factor.

<sup>c</sup> For peptide-specific IFN- $\gamma$  production, concentrations of peptides CEA, p53, and gp70 were 10, 2, and 1  $\mu\text{g/ml}$ , respectively. Each value represents IFN- $\gamma$  (pg/ml/10<sup>6</sup> cells/24 h).

<sup>d</sup> Three CEA-Tg mice/group were given MC38-CEA<sup>+</sup> tumors s.c. Eight days later mice were vaccinated with rV-CEA/TRICOM admixed with rF-GM-CSF. On day 14, tumors were irradiated (8 Gy). On days 15, 22, and 29, mice were boosted with rF-CEA/TRICOM admixed with rF-GM-CSF. Responses from pooled splenic T cells from cured mice were analyzed 6 months following the tumor transplant for CEA protein and p53 MHC-II-peptide-specific proliferation and CEA, p53, and gp70 peptide-specific IFN- $\gamma$  production. Control mice, normal age-matched CEA-Tg mice.

<sup>e</sup> Detection limit was 2.5 pg/ml.

CD3<sup>+</sup> T cells by immunohistochemistry (Fig. 3). Tumors treated with the combination of the CEA/TRICOM vaccine regimen in combination with radiation demonstrated a substantial increase of T cells in the tumor microenvironment as shown by CD3<sup>+</sup> T-cell staining by immunohistochemistry (Fig. 3D). This T-cell infiltration was confirmed by two-color flow cytometry (CD4/CD8). For these analyses, all of the cells were included, with tumor cells noted in the lower left quadrants, confirmed by staining with anti-CEA antibody (not shown; Fig. 3, E–H). Untreated tumors or tumors treated by radiation (8 Gy) demonstrated similar levels of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration (for CD8<sup>+</sup>, 7.1% and 7.2%; Fig. 3, E and G, respectively). Tumors treated with the CEA/TRICOM vaccine regimen demonstrated a slight increase in CD8<sup>+</sup> T-cell infiltration (11%; Fig. 3F). However, the combination of the CEA/TRICOM vaccine regimen with radiation resulted in a fivefold increase of infiltrating CD8<sup>+</sup> T cells (38%; Fig. 3H) over that induced by radiation alone. The T cell:tumor cell ratios for these groups were no treatment, 1:14; vaccine, 1:10; irradiation, 1:10; and vaccine in combination with radiation, 1:3.

**Cellular Immune Responses to Multiple Tumor Antigens after Combination Therapy with Vaccine and Radiation.** Cellular immune responses were monitored after treatment with the combination of vaccine therapy and radiation. MC38-CEA<sup>+</sup> tumor cells, in addition to expressing CEA, have been reported to express the gene products of murine retroviruses (30), including gp70 (28, 31), which could behave as targets of the CTL response. These cells also have been shown to overexpress wild-type p53, another potential target antigen (32). Tumor-bearing CEA-Tg mice treated with the combination of the CEA/TRICOM vaccine regimen and irradiation and cured of established tumor, as described previously, were monitored for 6 months. Control mice consisted of age-matched CEA-Tg mice that did not receive tumor and were not treated. At 6-month post-tumor cure, CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses specific for multiple TAA were measured from the tumor-free mice. As seen in Table 1, T cells from control CEA-Tg mice exhibited no increase in proliferative responses to CEA protein at any of the concentrations used. However, T cells from mice treated with the combination of the CEA/TRICOM vaccine regimen with radiation and cured of tumor demonstrated a 3.5-fold increase in CEA-specific proliferation, which was statistically significant ( $P = 0.02$ ), from responses noted from control T-cell responses. CD4<sup>+</sup> T-cell responses specific for an MHC class II restricted p53 peptide also were analyzed. A significantly higher proliferative response (3.2-fold;  $P = 0.007$ ) was observed from T cells of mice treated with the combination of vaccine and radiation as compared with the control group. T cells from either group failed to react with negative control protein, human serum albumin.

CD8<sup>+</sup> T-cell responses against CEA peptide then were monitored by measuring IFN- $\gamma$  production (Table 1). CD8<sup>+</sup> T cells from mice

treated with the combination of vaccine and radiation showed a much higher CEA-specific IFN- $\gamma$  production compared with the control group. To extend the findings to other potential TAAs, CD8<sup>+</sup> T-cell responses also were measured against an MHC class I restricted p53 or gp70 peptide. A much higher level of IFN- $\gamma$  production again was observed against the p53 and gp70 peptides from CD8<sup>+</sup> T cells from mice treated with the combination of vaccine and radiation. T cells from control mice demonstrated no reactivity to the CEA, p53, or gp70 peptides.

Because CEA is a self-antigen expressed in adult gastrointestinal tissue and to a lesser extent in other tissues of CEA-Tg mice, sera from CEA-Tg mice successfully treated for established CEA-expressing tumors by the CEA/TRICOM vaccination/radiation regimen after 6 months were examined for levels of ANA and antibodies specific for rheumatoid factor, nuclear ribonuclear protein, histone, topoisomerase-1 (scl-70), dsDNA, ssDNA, or circulating immune complexes. There were no detectable differences in sera samples from mice that received the combination therapy of CEA/TRICOM with radiation *versus* age-matched control mice.

**Characterization of MC38-CEA<sup>+</sup> Tumor Cells Defective for Fas Signaling.** To directly examine the contribution of Fas in the model described here, we generated MC38-CEA<sup>+</sup> cells that were defective for Fas signaling, designated MC38-CEA-DN1. To characterize these cells, Fas expression was monitored before and after radiation (Fig. 4B, *inset*) and compared with that of MC38-CEA<sup>+</sup> cells (Fig. 4A, *inset*). MC38-CEA-DN1 cells were 95% positive for surface expression of Fas before and after irradiation; however, the cell surface density (as measured by mean fluorescent intensity) increased by 20% following irradiation, likely because of up-regulation of endogenous Fas. Nonirradiated MC38-CEA<sup>+</sup> cells in comparison were weakly positive for Fas (8%), which increased to 80% positive following irradiation. To confirm whether the overexpressed truncated Fas was functionally active before and after 8 Gy radiation, MC38-CEA-DN1 cells were cultured in the presence of cross-linking anti-Fas mAb. Although the surface expression of Fas was high on the MC38-CEA-DN1 cells, they did not exhibit increased Fas-mediated lysis even after radiation (Fig. 4B), confirming the defect in the Fas signaling pathway. In contrast, after irradiation, MC38-CEA<sup>+</sup> cells demonstrated significant lysis in response to antibody-mediated Fas cross-linking (Fig. 4A). Functionality of the perforin pathway remained intact in MC38-CEA-DN1 cells, as demonstrated by the use of MC38-CEA-DN1 cell lines as target cells pulsed with the relevant (CEA) or irrelevant (VSVN) peptide in a 4-h CEA-specific CTL-mediated cytotoxicity assay (Fig. 4, C and D). In another experiment, the CTLs were preincubated with the perforin inhibitor CMA before they were incubated with the MC38-CEA<sup>+</sup> or MC38-CEA-DN1 target cells. The Ag-specific CTL-mediated lysis was blocked com-

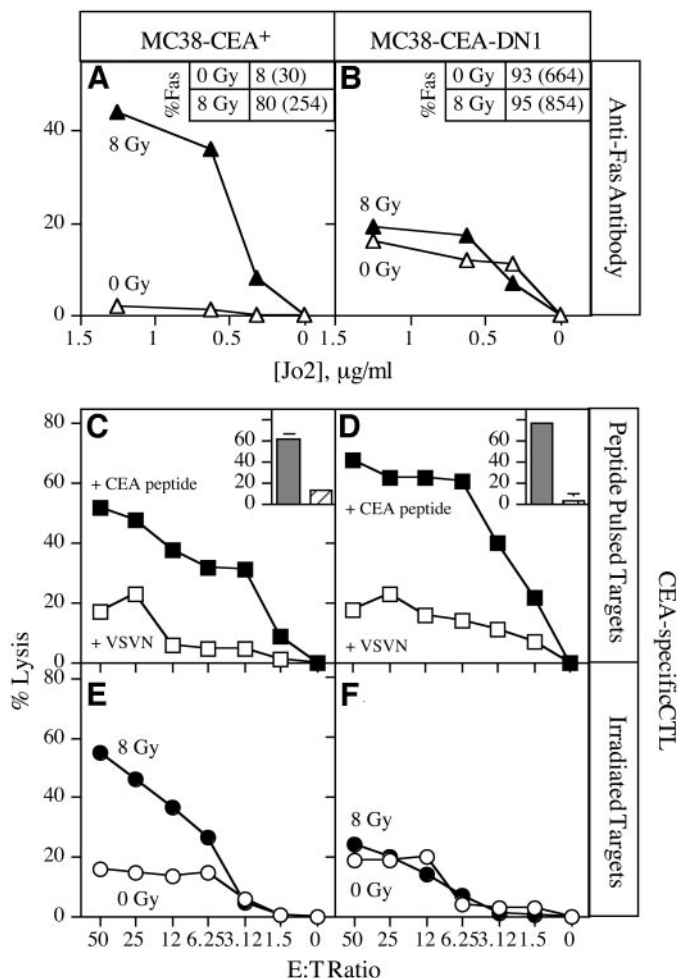


Fig. 4. Characterization of MC38-CEA<sup>+</sup> tumor cells and MC38-CEA-DN1 tumor cells defective for Fas signaling. MC38-CEA<sup>+</sup> tumor cells were transduced with a retrovirus encoding the gene for dominant-negative Fas (designated MC38-CEA-DN-1). These cells were compared with MC38-CEA<sup>+</sup> tumor cells for functional Fas activity, and carcino-embryonic antigen (CEA)-specific CTL sensitivity mediated by perforin or Fas. Functional Fas activity: MC38-CEA<sup>+</sup> (A) or MC38-CEA-DN-1 tumor cells (B) were irradiated (8 Gy; ▲) or not irradiated (△) and then recultured for 48 h. Cells then were analyzed for Fas-mediated killing by incubation with anti-Fas monoclonal antibody (Jo2) and protein G. *Inset*, depict Fas expression as determined by flow cytometric analysis. CTL sensitivity (perforin): MC38-CEA<sup>+</sup> (C) or MC38-CEA-DN-1 tumor cells (D) were irradiated (8 Gy) and recultured for 48 h. Tumor cells were pulsed with CEA peptide (■) or with control VSVN peptide (□) and coincubated with the indicated ratios of CEA-specific CTL for 4 h. *Inset*, depict lysis of tumor cells pulsed with CEA peptide and coincubated with a 50:1 ratio of CEA-specific CTLs (shaded bars) or CEA-specific CTLs that were pretreated with the perforin inhibitor concanmycin A (hatched bars). CTL sensitivity (Fas): MC38-CEA<sup>+</sup> (E) or MC38-CEA-DN-1 tumor cells (F) were irradiated (8 Gy; ●) or not irradiated (○), recultured for 48 h, and then coincubated with the indicated ratios of CEA-specific CTL for 18 h. Lysis was determined by <sup>51</sup>Cr release.

pletely after CMA pretreatment (Fig. 4, C and D, *inset*), further verifying that sensitivity to the perforin-mediated pathway was intact. Finally, MC38-CEA<sup>+</sup> or MC38-CEA-DN1 cells were irradiated and incubated with CEA-specific CTL (not CEA peptide pulsed). As reported previously and shown here, irradiated MC38-CEA<sup>+</sup> cells demonstrated increased sensitivity to CEA-specific CTL (Fig. 4E; Ref. 18). In contrast, irradiated MC38-CEA-DN1 cells were not efficiently lysed either before or after irradiation (Fig. 4F).

#### Role of Radiation Induced Fas Up-Regulation in Tumor Eradication by the Combination of Vaccine Therapy and Radiation.

We demonstrated that up-regulation of Fas on MC38CEA<sup>+</sup> tumor cells by irradiation in conjunction with vaccine therapy resulted in the delay of tumor progression and eventual tumor resolution by the endogenous host response (Fig. 2, D and H). To determine directly

whether radiation-induced Fas up-regulation on MC38-CEA<sup>+</sup> cells *in vivo* was responsible for tumor regression, groups of CEA-Tg mice were transplanted with either MC38-CEA<sup>+</sup> or MC38-CEA-DN1 tumors (Fig. 5). When MC38-CEA<sup>+</sup> tumors were treated with the combination of the CEA/TRICOM vaccine regimen with radiation, there was a notable significant delay in tumor progression ( $P = 0.0003$  versus no treatment;  $P = 0.006$  versus vaccine or irradiation alone), with 50% of the mice becoming tumor free (Fig. 5D). MC38CEA<sup>+</sup> tumors that were not treated (Fig. 5A), treated with the CEA/TRICOM vaccine regimen alone (Fig. 5B), or treated by irradiation alone (Fig. 5C) grew at comparable rates. Similarly, MC38-CEA-DN1 tumors that were not treated (Fig. 5E), treated with the CEA/TRICOM vaccine regimen alone (Fig. 5F), or treated by irradiation alone (Fig. 5G) grew at comparable rates. Treatment of MC38-CEA-DN1 tumors with the combination of the CEA/TRICOM vaccine regimen with radiation failed to mediate tumor regression (Fig. 5H;  $P = 0.8$  versus no treatment), defining a crucial requirement in this model system for the Fas signaling pathway.

#### DISCUSSION

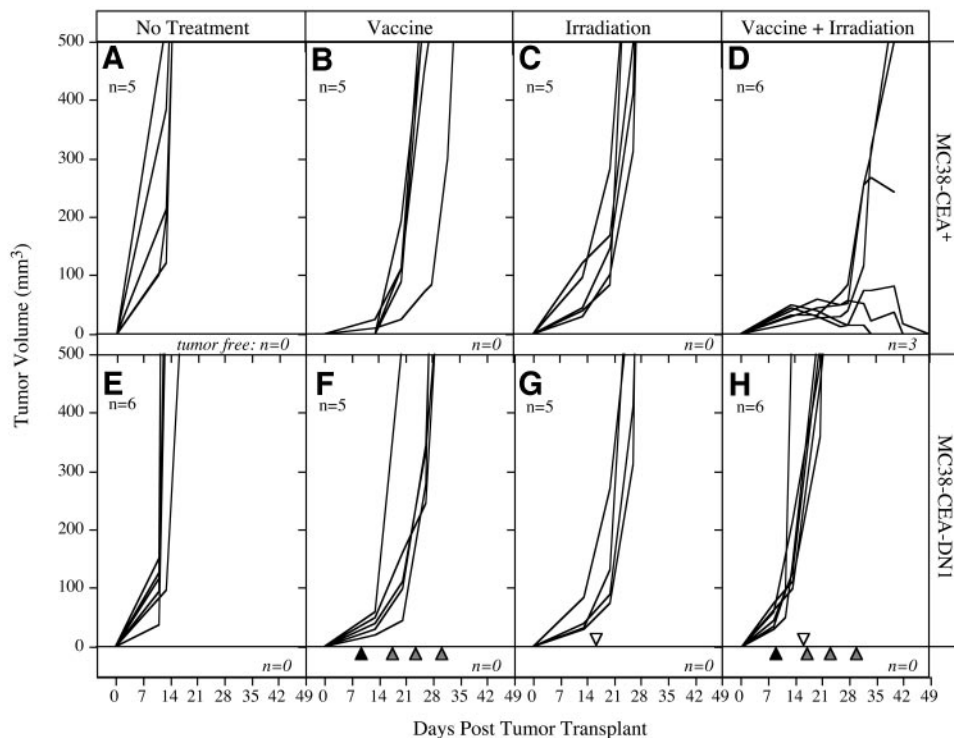
Ionizing radiation is a primary modality in cancer therapy. The “curative” doses of radiation used clinically are typically in the higher range and induce tumor regression directly. However, sublethal doses of radiation have been associated with the up-regulation of several classes of molecules on tumor cells that could potentially influence the immune system, including HLA molecules, costimulatory molecules, adhesion molecules, tumor-associated antigens, heat-shock proteins, inflammatory mediators, cytokines, and death receptors (for review, see Ref. (3)). Of the up-regulated death receptors, perhaps the most characterized is Fas. Fas has been shown to be up-regulated after irradiation using a variety of human tumor cell lines: breast carcinoma and osteosarcoma (5), colorectal carcinoma (33), lymphoma (34), and squamous cell carcinoma (35, 36).

Fas and its cognate ligand, FasL, are transmembrane glycoproteins belonging to the TNF receptor and ligand superfamilies, respectively. Engagement of Fas by FasL triggers the recruitment of adaptor proteins, followed by the activation of the caspase-signaling pathway, culminating in physiologic cell death. Functional Fas is highly expressed on a variety of nonmalignant tissues; however, the role of Fas on solid tumor cells remains to be elucidated with regard to its role as a target of endogenous immune-mediated effector mechanisms or its practical suitability as a mechanism for enhancing the effects of anticancer therapies.

The model system here involved a murine adenocarcinoma cell line, MC38-CEA<sup>+</sup>, that expressed low levels of Fas (Fig. 1A) and was weakly sensitive to Ag-specific CTL (Fig. 4A). Perforin-mediated cytotoxicity and Fas/FasL interactions have been characterized as the major effector mechanisms for CTL function (37). The CEA<sub>526</sub> CTL line used here can lyse CEA peptide-pulsed targets through perforin mechanisms (Fig. 4C). However, CTL killing of MC38-CEA<sup>+</sup> expressing endogenous Ag was not significant through perforin, which might reflect the relatively lower strength of signal-1 (*i.e.*, MHC/Ag recognition). This is consistent with the observation of Kessler *et al.* (38), who demonstrated that T-cell receptor signaling that was too weak to elicit perforin-dependent cytotoxicity or cytokine production could induce Fas-dependent cytotoxicity, possibly by translocation of preformed FasL to the cell surface.

It was demonstrated previously that irradiation of an 8-day s.c. tumor (8 Gy) in combination with the adoptive transfer of CEA-specific CTL resulted in significant antitumor effects, whereas radiation alone or CTL adoptive transfer alone had no effect on tumor growth. Because adoptive T-cell therapy is an extremely difficult and

Fig. 5. Role of radiation-induced Fas up-regulation in tumor eradication by the combination of vaccine therapy and radiation. Carcinoembryonic antigen (CEA)-Tg mice were injected with MC38-CEA<sup>+</sup> tumor cells s.c. (A–D) or MC38-CEA-DN-1 tumor cells s.c. (E–H). A and E, mice received no treatment. B and F, mice were vaccinated with recombinant vaccinia (rV)-CEA/triad of costimulatory molecules (TRICOM) on day 8 (▲), followed by boosting with recombinant fowlpox (rF)-CEA/TRICOM on days 15, 22, and 29 (gray triangles). C and G, tumors in mice were subjected to external-beam irradiation (8 Gy) *in situ* on day 14 (▽). D and H, mice were vaccinated with rV-CEA/TRICOM on day 8 (▲). Tumors were subjected to external-beam irradiation (8 Gy) *in situ* on day 14 (▽), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). All of the vaccines were coadministered with rF-granulocyte macrophage colony-stimulating factor. Tumor volume was monitored.



costly modality of cancer therapy and is in effect passive therapy, we sought to induce CEA-specific T cells via active specific immunotherapy, priming CEA-Tg mice with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM, and combining this modality with external-beam irradiation. In this study, it was shown that sublethal doses of radiation up-regulated cell surface expression of Fas, ICAM-1, and MHC class I on MC38-CEA<sup>+</sup> tumors in a dose-dependent manner, as determined by flow cytometry (Fig. 1). The up-regulation of MHC class I on human and mouse tumor cells *in vitro* has been reported in various studies following 10–100 Gy of radiation (39, 40). In addition, overexpression of ICAM-1 on tumor cells has been associated with increased sensitivity to CTL-mediated lysis (41). It was shown here by immunohistochemistry and confirmed by flow cytometry that radiation-induced Fas up-regulation persisted on MC38-CEA<sup>+</sup> tumors for >11 days. This observation extends that of Morgan *et al.* (42), who demonstrated that cell lines developed from Chinese hamster ovary cells subjected to 15 Gy radiation exhibited heritable nonlethal effects that were maintained for 80-cell generations.

CEA-Tg mice were vaccinated with a diversified prime and boost regimen using rV-CEA/TRICOM/rF-CEA/TRICOM in combination with sublethal irradiation of the tumor. The advantages of the diversified vaccine prime and boost regimen (rV prime followed by rF boost) have been described previously (12–14), as has the use of T-cell costimulation via inserting multiple costimulatory molecules into these vectors (15–17). Mice were transplanted s.c. with MC38-CEA<sup>+</sup> tumor cells, and after 8 days, the mice were primed with rV-CEA/TRICOM. Tumors were subjected to one dose (8 Gy) of irradiation. Mice were boosted with rF-CEA/TRICOM on days 15, 22, and 29. This dose and schedule of radiation were determined to be noncurative; the MC38-CEA<sup>+</sup> tumors exhibited a marginal but insignificant reduction in growth as compared with nonirradiated tumors (Fig. 2C). The vaccine regimen alone did not affect the growth rate of the transplanted tumor. Although this vaccine regimen shows antitumor effects when begun 4 days post-tumor transplant, the 8-day tumor model was selected for its lack of response to vaccine alone. However, the combination of the vaccine regimen with radiation in the 8-day

model resulted in a significant reduction of tumor volume. In addition, 50% of the mice in this treatment group completely eradicated the tumor mass (Fig. 2D and Fig. 5D).

Clinically, irradiation of tumors is routinely fractionated into relatively small, daily doses as opposed to a single large dose because of potential coincidental damage to normal tissues. To determine the effects of fractionated radiation on Fas up-regulation, MC38-CEA<sup>+</sup> tumor cells or s.c. transplanted MC38-CEA<sup>+</sup> tumors were subjected to 8 Gy radiation delivered as 2 Gy/day for 4 days. The up-regulated expression level of the Fas molecule following the fractionated radiation schedule was shown to be comparable with that of the single 8-Gy dose (Fig. 2, D and H). This is in agreement with the observations of Sheard *et al.* (33), who using human colorectal, breast, cervical, and osteosarcoma cell lines demonstrated that Fas was similarly up-regulated after irradiation whether delivered as a single dose of 16 Gy or fractionated to 2 Gy/day for 8 days. As seen with the combination of the vaccine regimen with single-dose radiation, the combination of the vaccine regimen with fractionated radiation resulted in a significant reduction of tumor volume, with 56% of the mice resolving their tumor burden (Fig. 2H). The role of Fas in tumor therapy *in vivo* was directly confirmed by using MC38-CEA-DN1 cells (MC38-CEA<sup>+</sup> cells stably transfected with Fas lacking the intercellular signaling domain), which were not successfully treated with the combination of the vaccine regimen and radiation (Fig. 5H; Ref. 20).

MC38-CEA<sup>+</sup> tumor destruction in response to the combination of the vaccine regimen with radiation was shown to be associated with a massive infiltration of CD8<sup>+</sup> T cells, which was confirmed by immunohistochemistry and flow cytometry (Fig. 3). This finding is intriguing and could possibly indicate the mechanism of tumor destruction after combination therapy. This suggestion is supported by the enhanced efficiency of killing *in vitro* of Fas-up-regulated MC38-CEA<sup>+</sup> tumor cells by CEA-specific T cells (Fig. 4E). Together, these data demonstrate that up-regulated Fas on tumor cells can be a target of T cells *in vivo*. The mechanism for the increase in infiltrating T cells into the Fas-overexpressing tumor sites remains to be elucidated.



However, these data are consistent with those reported by Ganss *et al.* (43), who found whole-body lethal irradiation resulted in remodeling of the tumor vasculature, permitting T-cell access and subsequent tumor therapy.

MC38-CEA<sup>+</sup> tumor cells, in addition to expressing CEA, have been noted to overexpress other TAAs, such as wild-type p53, which can be driven to even higher expression levels with radiation (32). These tumor cells also express potential immunoreactive antigens from endogenous germline retroviral encoded proteins, including gp70, which could contribute to an immune response (28, 31). Yang *et al.* (28) have demonstrated that adoptive transfer of CTL specific for gp70 to mice was sufficient for therapy of several types of metastases. It here was shown that mice cured of MC38-CEA<sup>+</sup> tumors by the combination of the vaccine regimen and radiation generated CD4<sup>+</sup> T-cell responses against not only CEA but also against p53 (Table 1). In addition, CD8<sup>+</sup> T-cell responses specific for p53 and gp70 were detected. Because p53 and gp70 were not encoded by the vaccine regimen, it can be inferred that these antigens were donated from the tumor itself. Interestingly, the IFN- $\gamma$  response against gp70 was 15-fold greater than that against the CEA peptide, and it can be postulated that the majority of the antitumor effect was mediated by gp70 responses. Preliminary data indicate that gp70-specific CD8<sup>+</sup> T-cells are detectable in the tumors of mice treated with the combination of the vaccine regimen with radiation on day 21 (data not shown). Studies are ongoing to determine whether these cells are involved in the antitumor effects observed.

Antigens from peripheral tumor cells can enter the MHC class I pathway for presentation by host antigen-presenting cells to CD8<sup>+</sup> T cells via a process commonly described as "cross-presentation" (44). It has been suggested that drug therapy protocols using apoptosis-inducing agents could prime antitumor immune responses. Nowak *et al.* (44), in a murine model, demonstrated that tumor cell apoptosis *in vivo* induced by the chemotherapeutic drug gemcitabine increased tumor antigen cross-presentation, which subsequently led to priming of tumor-specific CD8<sup>+</sup> T cells. Pilon *et al.* (45) suggested that immunization with dominant TAA vaccines, followed by a general enhancement of CD4<sup>+</sup> T-cell activity to multiple TAA via cross-priming, might be responsible for long-term tumor protection in a HER-2/*neu* mouse model. We have shown that vaccination using the CEA/TRICOM regimen induced CEA-specific T-cell responses (Table 1). Local irradiation of the tumor mass, in addition to up-regulating Fas (Fig. 1), could have induced apoptosis of a fraction of the tumor cells, thus providing a "boost" to the CEA-specific T cells, while inducing T-cell responses to additional tumor antigens such as p53 and gp70 through cross-priming (Table 1). This "antigen cascade" would be further augmented by Fas-mediated destruction of tumor cells.

In addition to radiation, many chemotherapeutic agents used as "standard of care," such as gemcitabine (46), 5-fluorouracil (47), cisplatin (47), CPT-11 (47), and others (48), also have been shown to enhance Fas expression. Thus, these observations support the notion that strategies such as targeted radiation or chemotherapy aimed at inducing Fas expression on tumor cells may be used to improve antitumor responses via vaccine therapy (36, 48). It has been demonstrated in preclinical (16, 49) and now clinical (50) settings that a diversified vaccination regimen (primary vaccination with rV-CEA/TRICOM) followed by boosting with rF-CEA/TRICOM was optimal in the induction of CEA-specific T-cell responses. Taken collectively, these results suggest that strategies directed at preferential induction of Fas on tumor cells via localized external-beam radiation in combination with active-specific immunotherapy may induce far more effective antitumor responses than those seen using either modality alone.

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