Irradiation of Tumor Cells Up-Regulates Fas and Enhances CTL Lytic Activity and CTL Adoptive Immunotherapy

Mala Chakraborty, Scott I. Abrams, Kevin Camphausen, Kebin Liu, Tamalee Scott, C. Norman Coleman and James W. Hodge

_J Immunol_ 2003; 170:6338-6347; doi: 10.4049/jimmunol.170.12.6338
http://www.jimmunol.org/content/170/12/6338

References
This article cites 57 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/170/12/6338.full#ref-list-1

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Irradiation of Tumor Cells Up-Regulates Fas and Enhances CTL Lytic Activity and CTL Adoptive Immunotherapy

Mala Chakraborty,* Scott I. Abrams,* Kevin Camphausen,† Kebin Liu,* Tamalee Scott,† C. Norman Coleman,† and James W. Hodge1*

CD8+ CTL play important roles against malignancy in both active and passive immunotherapy. Nonetheless, the success of antitumor CTL responses may be improved by additional therapeutic modalities. Radiotherapy, which has a long-standing use in treating neoplastic disease, has been found to induce unique biologic alterations in cancer cells affecting Fas gene expression, which, consequently, may influence the overall lytic efficiency of CTL. Here, in a mouse adenocarcinoma cell model, we examined whether exposure of these tumor cells to sublethal doses of irradiation 1) enhances Fas expression, leading to more efficient CTL killing via Fas-dependent mechanisms in vitro; and 2) improves antitumor activity in vivo by adoptive transfer of these Ag-specific CTL. Treatment of carcinoembryonic Ag-expressing MC38 adenocarcinoma cells with irradiation (20 Gy) in vitro enhanced Fas expression at molecular, phenotypic, and functional levels. Furthermore, irradiation sensitized these targets to Ag-specific CTL killing via the Fas/Fas ligand pathway. We examined the effect of localized irradiation of s.c. growing tumors on the efficiency of CTL adoptive immunotherapy. Irradiation caused up-regulation of Fas by these tumor cells in situ, based on immunohistochemistry. Moreover, localized irradiation of the tumor significantly potentiated tumor rejection by these carcinoembryonic Ag-specific CTL. Overall, these results showed for the first time that 1) regulation of the Fas pathway in tumor cells by irradiation plays an important role in their sensitization to Ag-specific CTL; and 2) a combination regimen of tumor-targeted irradiation and CTL promotes more effective antitumor responses in vivo, which may have implications for the combination of immunotherapy and radiation therapy. The Journal of Immunology, 2003, 170: 6338–6347.

CD8+ CTL have been described to play crucial roles in host defense against malignancies in both mouse and human studies (1–4). However, it is also becoming more clear that neoplastic cells may evade or resist adaptive immune responses at multiple levels within the effector-target interaction (1, 5–13). Thus, we have been interested in exploring fundamental aspects of the T cell-tumor cell interaction, particularly in the area of carcinoma, which may have implications for designing improved immunotherapeutic strategies. One possibility involves the use of localized, external beam irradiation of solid tumors in concert with active or adoptive immunotherapy approaches in preclinical models.

The conventional reasoning behind the use of radiation therapy has been to exploit its cytotoxic properties. However, recent studies in vitro support the idea that irradiation may also induce immune modulatory effects, such as up-regulation of cell surface expression of MHC determinants, adhesion/costimulatory molecules, or Fas (CD95) on hematologic or nonhematologic cells (12, 14–18). Additionally, others (19, 20) have reported that local or sublethal, whole-body radiation can be used in combination with immunotherapy to elicit more favorable antitumor responses in vivo; however, the mechanisms underlying those outcomes remain unclear. Of particular interest in this study was the potential effect of irradiation on the regulation of the Fas pathway in murine colon carcinoma cells and its consequent role in Ag-specific CD8+ CTL killing.

The Fas/Fas ligand (FasL)2 system has been characterized as an integral process for the maintenance of immune privilege and the regulation of immune homeostasis of peripheral lymphoid interactions under both normal and pathologic conditions (21–27). Moreover, in the area of lymphocyte-mediated cytotoxicity, the induction of Fas expression on tumor cells may enable their destruction by Ag-specific immune effector cells via Fas-dependent mechanisms. In mouse models (28–32) it has been demonstrated that CD8+ CTL mediate lysis of susceptible targets via two major effector mechanisms following MHC/Ag recognition: 1) secretion of perforin and granzyme contents, and 2) engagement of Fas by FasL expressed by the activated CTL. Both pathways lead to apoptotic cell death involving caspase-dependent and/or -independent signaling events (28, 32). The contribution of each effector mechanism to the overall lytic response probably reflects, or depends upon, intrinsic characteristics of the given target cell population.

Here we examined whether potential changes in the regulation of Fas expression and function of tumor cells by gamma irradiation might then improve antitumor CTL responses in vitro and in vivo. To that end, we made use of a carcinoembryonic Ag (CEA)-specific CD8+ CTL-tumor model previously established in our laboratory (33). Because CEA expression is prevalent among diverse human carcinomas, namely colorectal, gastric, pancreatic, breast, and non-small cell lung malignancies (34–36), transgenic mice expressing the human CEA gene (37) were used in our laboratory as a more relevant preclinical model to investigate various experimental immunotherapies (38). CEA-specific, H-2Db-restricted...
CTL were produced from these CEA-transgenic mice by immunization with recombinant poxvirus-based vectors (33). A relevant epitope peptide was identified, termed CEA260, which was subsequently employed for in vitro expansion and establishment of a CTL line. CTL propagated with peptide-mediated specific lysis of syngeneic MC38 colon carcinoma cells endogenously expressing (via retroviral transduction) human CEA. Using this CEA model system, we have now examined the immunobiologic consequences of sublethal doses of tumor-targeted irradiation on 1) CEA, MHC class I, ICAM-1, and Fas expression in vitro; 2) CTL lytic efficiency and the role of endogenous Fas/FasL interactions in tumor cell destruction in vitro; and 3) Fas expression in vivo and the efficacy of CTL adoptive immunotherapy.

Overall, these results revealed for the first time that regulation of the Fas pathway in tumor cells by sublethal irradiation significantly improved their lytic susceptibility to Ag-specific CTL, and that a therapeutic schedule consisting of localized radiation of s.c. growing tumors, followed by systemic CTL adoptive transfer led to a synergistic inhibition of tumor growth. Thus, irradiation 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-2b), has been described previously (39). MC38 cells expressing human CEA were generated by retroviral transduction with CEA cDNA (39) and are designated MC38-CEA+. The EL-4 cell line (H-2b) was obtained from American Type Culture Collection (Manassas, VA). The RMA lymphoma cell line (H-2b) was provided by Dr. J. W. Yewdell (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD).

T cell lines

The H-2Dd-restricted, CEA-specific CD8+ CTL line, designated CEA260, was generated from CEA-Tg mice (C57BL/6 background, H-2b) as previously described (33) and recognizes the peptide epitope CEA260–263 (EAQNNTTYL). The H-2Dd-restricted, p53 peptide-specific CD8+ CTL line, designated p53262, was generated from C57BL/6 mice as previously described (40) and recognizes the peptide epitope p53262–265 (KYMNC55 SCM). The H-2Dd-restricted, FluNP peptide-specific CD8+ CTL line, designated FluNPp12, was generated from C57BL/6 mice as previously described (40) and recognizes the peptide epitope FluNPp12–240 (AYKMC55). The CEA 526 CTL were recovered on day 6 of the stimulation cycle by using the ThermoScript RT-PCR system (Invitrogen, San Diego, CA).

Cytotoxicity assays

The CEA260 CTL were recovered on day 6 of the stimulation cycle by centrifugation through a density gradient (LSM, Organon Teknika, West Chester, PA). EL-4 cells were prepared for use as targets in a standard cytotoxicity assay using 51Cr as previously described (40). These irradiated cells (5 × 103 cells/well) were incubated with 1 μg/ml CEA260 peptide or vesicular stomatitis virus-NrLS (RGYVYQGL) (42) as a negative control and coincubated with CEA260 CTL at various E:T cell ratios ranging from 100:1 to 12.5:1 in 96-well U-bottom plates (Costar, Cambridge, MA), and incubated for 18 h at 37°C with 5% CO2. MC38 or MC38-CEA+ tumor cells were sham-irradiated (0 Gy) or irradiated (10–50 Gy) and recultured for 24 h, then prepared for use as targets using 51Cr. CEA260 CTL and targets (5 × 103 cells/well) were suspended in complete medium, combined at E:T cell ratios ranging from 100:1 to 12.5:1 in 96-well U-bottom plates (Costar, Cambridge, MA), and incubated for 18 h at 37°C with 5% CO2, since the time-course experiments revealed optimal lysis against these targets under longer incubation conditions as previously described (33). After incubation, supernatants were collected using a Supernatant Collection System (Skatron, Sterling, VA), and radioactivity was quantitated using a gamma counter (Cobra Autoy; Packard, Downers Grove, IL). The percentage of specific release of 51Cr was determined by the standard equation:

\[ \text{Specific lysis} = \left( \frac{\text{Experimental spontaneous}}{\text{Maximum spontaneous}} \right) \times 100 \]

where indicated, CTL activity was converted to lytic units (LU) as described by Wunderlich et al. (43). In other experiments EL-4 cells and RMA cells were prepared for use as targets in a similar manner. In the indicated experiments neutralizing mAbs for Fasl (clone MFL3) or ICAM-1 (clone 3E2; BD PharMingen) were added to the cytotoxic assay to ascertain the roles of these molecules in CTL killing.

Functional Fas assay

MC38 and MC38-CEA+ tumor cells were nonirradiated (0 Gy) or were irradiated (20 Gy) and recultured for 24 h. Cells were then labeled with 35Cr as described above and were analyzed for Fas-mediated killing by incubating 8×103 Fasl-Ab mAb (1D3, BD PharMingen) plus protein G (10 μg/ml; Amersham Pharmacia Biotech, Uppsala, Sweden) to maximize cross-linking of the primary mAb. Control wells consisted of tumor cells incubated with isotype-matched Ab. RMA cells were used as a positive control for Fas-mediated cytotoxicity in this assay format.

Animals

For in vivo studies, 6- to 8-wk-old female C57BL/6 mice were used (National Cancer Institute, Frederick, MD). C57BL/6 mice transgenic for human CEA (designated CEA-Tg) were originally obtained from a breeding pair provided by Dr. J. Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of the CEA-Tg mouse have been previously described (37). PCR of DNA from whole blood to detect the CEA gene was used to screen for CEA-positive mice as previously described (44). Mice were housed and maintained under pathogen-free conditions in microisolator cages.

Flow cytometric analysis

Cell surface staining was performed with primary FITC-labeled mAb COL-1 (anti-CEA (41), H-2Kd, H-2Dd, and ICAM-1 (CD54)), Fas (CD95) cell surface staining was performed with primary FITC- or PE-labeled mAb. All mAb were purchased from BD PharMingen (San Diego, CA). Cell fluorescence was analyzed and compared with the appropriate isotype-matched controls (BD PharMingen) with a FACScan cytometer using LYSIS II software (BD Biosciences, Mountain View, CA). Dead cells were electronically excluded from analysis based on propidium iodide exclusion. In experiments examining cell cycle in vitro-stimulated cells were processed using Vybrant CFDA SE Cell Tracker Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions before irradiation and cytometric analysis.

RT-PCR detection of Fas and ICAM-1

Total RNA was isolated from tumor cells (irradiated or nonirradiated) using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions for first-strand cDNA synthesis using the ThermoScript RT-PCR system (Invitrogen, San Diego, CA). The cDNA was then used as templates for PCR amplification of mouse Fas, ICAM-1, and β-actin (30 cycles). The PCR primers for mouse Fas were: forward primer, 5′-ATGCTGTGATCTGGCT-3′; and reverse primer, 5′-TCACTCCAGACATGTGTC-3′. The PCR primers used for ICAM-1 were: forward primer, 5′-CAGATGCCGACCAGGAG-3′; and reverse primer, 5′-ACAGACTTCACACCCCCAGATG-3′. The PCR primers for mouse β-actin were: forward primer, 5′-ATTGTTACCAACTGGGAC-3′; and reverse primer, 5′-CTCTATGAGGAATCTGTGAC-3′. Densitometric analysis was performed on a Macintosh computer using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).
Tumor therapy studies

Before transplantation to mice, MC38-CEA cells were trypsinized, dispersed through a 70-μm pore size cell strainer (Falcon; BD Biosciences, Franklin Lakes, NJ), and washed twice in HBSS before final suspension in HBSS. Mice were injected with \(3 \times 10^5\) MC38-CEA tumor cells s.c. in the quadriceps area of the right hindlimb. Nine days following tumor transplant, tumors were irradiated with 8 Gy. In a subset of mice, tumors were excised 72 h following tumor irradiation, fixed, sectioned at 5 μm, and stained with anti-Fas mAb or an isotype control for immunohistochemical analysis. In another subset of mice, 24 h following tumor irradiation, mice received \(8 \times 10^6\) CEA526 T cells by adoptive transfer i.v. Control mice received \(8 \times 10^6\) FluNP366 T cells by adoptive transfer i.v. 24 h following tumor irradiation. This CTL line (FluNP366) specifically lysed FluNP366 peptide-pulsed EL-4 cells in a 4-h assay (90% at a 5:1 E:T ratio). In addition, this CTL line did not significantly lyse nonirradiated or irradiated MC38-CEA tumors cells (data not shown). Tumors were measured daily by digital caliper in two dimensions, and the volumes were calculated as previously described (45). Animals were sacrificed when any tumor measurement (length or width) exceeded 20 mm3.

Statistical analysis of the data

Where indicated, the results of tests of significance are reported as \(p\) values and are derived from Student’s \(t\) test using a two-tailed distribution. The \(p\) values were calculated at 95% using the StatView 4.1 software package (Abacus Concepts, Berkeley, CA).

**FIGURE 1.** Irradiation increases tumor cell sensitivity to Ag-specific cytotoxic T cell killing. A, \(^{51}\)Cr-labeled EL-4 cells pulsed with CEA526 peptide (●) or EL-4 cells pulsed with vesicular stomatitis virus-N52–59 peptide (○) were coincubated with the indicated ratios of CEA-specific CTL for 4 h. B, \(^{51}\)Cr-labeled MC38 tumor cells (□) or MC38 tumor cells expressing CEA (MC38-CEA*; ○) were coincubated with the indicated ratios of CEA-specific CTL for 18 h. C, MC38 (■) or MC38-CEA* cells (○) were irradiated at a dose of 20 Gy and recultured for 24 h. Cells were then labeled with \(^{51}\)Cr and were coincubated with the indicated ratios of CEA-specific CTL for 18 h. The nonspecific lysis ranged between 11–16% for all groups. This experiment was repeated five times with similar results.

**FIGURE 2.** Tumor irradiation up-regulates Fas and ICAM-1 expression in a dose-dependent manner. MC38 cells were irradiated at doses from 0–50 Gy and recultured for 48 h. Cells were then analyzed for Fas by flow cytometry (A). Depicted is Fas expression after exposure to 0 Gy (dashed histogram) or 20 Gy (solid histogram). Inset numbers are the percentage of positive cells (mean fluorescent intensity). Staining with isotype control Ab is indicated by the shaded histogram. Cells were further analyzed for Fas (C, ○), ICAM-1 (C, △), MHC-I (E, ◆), and CEA (E, □). MC38-CEA* tumor cells were also irradiated at doses from 0–50 Gy and recultured for 48 h. Cells were then analyzed for Fas by flow cytometry (B). Depicted is Fas expression after exposure to 0 Gy (dashed histogram) or 20 Gy (solid histogram). Staining with isotype control Ab is indicated by the shaded histogram. Cells were further analyzed for Fas (D, ●), ICAM-1 (D, △), MHC-I (F, ◆), and CEA (F, □). Up-regulation of Fas and ICAM-1 after irradiation was confirmed by PCR (G). These experiments were repeated three times with similar results.

**Results**

**Irradiation of tumor cells increases their lytic sensitivity to Ag-specific CTL**

Studies suggest that ionizing radiation mediates multiple biologic or immunologic consequences against neoplastic cells, ranging from effects on Ag expression to induction of apoptotic cell death.
Here, we examined whether sublethal doses of irradiation of Ag-bearing tumor cells improves the lytic efficiency of Ag-specific CTL. To explore this idea, we made use of a mouse Ag-specific CTL/colon carcinoma cell model. Fig. 1A illustrates the Ag specificity of the CTL line against EL-4 targets pulsed with the relevant (CEA) or irrelevant (vesicular stomatitis virus) peptide. In regard to the radiation studies, MC38 and MC38 cells, employed as an Ag-negative control, were either irradiated at 20 Gy or sham-irradiated, and then were used as targets for lysis by CEA-specific CTL. In the absence of irradiation there was a moderate amount of lysis of MC38-CEA+ cells by CEA-specific CTL compared with MC38 cells (Fig. 1B; LU = 5.1 for MC38-CEA+ vs >2 for MC38 tumor targets). However, after irradiation, there was a significant increase in lysis of MC38-CEA+ cells at the highest E:T cell ratio (Fig. 1B; p = 0.003; LU = 20 for irradiated target cells), with high levels of lysis observed over several E:T cell ratios. In contrast, irradiation failed to increase lysis of the MC38 cells at any E:T cell ratio.

Tumor irradiation up-regulates Fas and ICAM-1 expression in a dose-dependent manner

Next, we examined cell surface expression of several phenotypic markers on MC38-CEA+ following irradiation, which may have impacted CTL lytic efficiency. MC38 and MC38-CEA+ cells were irradiated at several doses (0–50 Gy), and cell surface expression of Fas, ICAM-1, CEA, and MHC class I molecules (e.g., H-2Kb) was monitored by flow cytometry after 48 h. For these analyses, the populations of cells that were positive for propidium iodide staining never exceeded 5%, and these dead cells were electronically excluded from analysis. Irradiation induced significant expression of Fas on both MC38 and MC38-CEA+ cells in a dose-dependent manner (Fig. 2, A–D). The maximum up-regulation of Fas was noted at 10–20 Gy. MC38 and MC38-CEA+ cells subjected to 8 Gy irradiation expressed Fas at levels of 30 and 25%, respectively (not shown). In addition, irradiation increased the expression of ICAM-1 on both MC38 and MC38-CEA+ cells in a dose-dependent manner, albeit to a lesser extent than Fas. In contrast, there were no detectable changes in the expression levels of MHC class I or CEA determinants after irradiation in both cell lines (Fig. 2, E and F). Fas up-regulation in response to irradiation was confirmed at the molecular level by RT-PCR, followed by densitometric analysis (Fig. 2G). MC38 cells irradiated with 10 or 20 Gy had a 3-fold increase in Fas mRNA compared with unirradiated MC38 cells. MC38 cells irradiated with 50 Gy up-regulated Fas mRNA by 5-fold. Similarly, MC38-CEA+ tumor cells irradiated with 10, 20, or 50 Gy up-regulated Fas mRNA by 4-, 3-, and 5-fold, respectively. The up-regulation of ICAM-1 in response to irradiation was also monitored by RT-PCR (Fig. 2G). Although MC38 or MC38-CEA+ tumor cells did not express detectable levels of ICAM-1 mRNA after irradiation at 10, 20, or 50 Gy, ICAM-1 mRNA was induced. The dose of radiation chosen for the remaining in vitro studies was based on determination of the optimal dose of radiation required to up-regulate Fas (20 Gy).

**Irradiation-induced Fas up-regulation is biologically active**

We found that irradiation sensitized MC38-CEA+ cells, but not MC38 cells, to Ag-specific CTL-mediated killing (Fig. 1). However, Fas was up-regulated to similar levels on both cell lines after irradiation (Fig. 2, A–D and G). These findings prompted us to investigate the functional status of Fas in both cell lines before and after irradiation. MC38 and MC38-CEA+ cells were irradiated (20 Gy), and then cultured in the presence of an anti-Fas mAb under agonistic conditions (see Materials and Methods). Fas-sensitive RMA cells were used as a positive control in this assay format, and under these conditions, anti-Fas mAb efficiently mediated lysis of RMA cells (Fig. 3A). Furthermore, irradiated, but not unirradiated, MC38 cells underwent significant (p = 0.0001 at 0.1 μg/ml Jo2) lysis in response to anti-Fas stimulation (Fig. 3B). Similarly, irradiated, but not unirradiated, MC38-CEA+ cells underwent Fas-mediated lysis (Fig. 3C). To confirm the specificity of anti-Fas mAb-mediated lysis, an isotype-matched control Ab also was used (Fig. 3A, ◯), which failed to induce lysis in all groups (data not shown).

**Increased tumor cell sensitivity to CTL killing after irradiation is blocked by anti-FasL**

The phenotypic changes noted after irradiation of MC38 and MC38-CEA+ tumor cells included the up-regulation of Fas (Figs. 2 and 3) and ICAM-1 (Fig. 2). To determine the functional roles of Fas and ICAM-1 in the CTL lytic process, neutralizing mAb directed against FasL or ICAM-1 were added to the assay. (The anti-Fas mAb was not tested because of potential concern of inducing, rather than inhibiting, lytic activity.) Anti-ICAM-1 mAb did not appear to block CTL-mediated lysis of either unirradiated (Fig. 4A) or irradiated (Fig. 4B) MC38-CEA+ cells compared with the isotype-matched control. In contrast, anti-FasL mAb completely inhibited CTL-mediated lysis of unirradiated or irradiated
This experiment was repeated four times with similar results. MC38-CEA+ tumor cells were irradiated (20 Gy; □) or nonirradiated (○), recultured for 24 h, and then coincubated with the indicated ratios of CEA-specific CTL for 18 h in the presence of isotype control Abs (A). A–C: Abs were used at 10 μg/ml. D: Irradiated MC38-CEA+ tumor cells (●) were coincubated with CEA-specific CTL in the presence of blocking Abs (A–C) or FasL (□). MC38-CEA+ tumor cells were also coincubated with CEA-specific CTL in the presence of blocking Abs (A–C) or FasL (□). A, C, and D: Abs were used at 10 μg/ml. D: Irradiated MC38-CEA+ tumor cells (●) were coincubated with CEA-specific CTL in the presence of blocking Abs (A–C) or FasL (□). This experiment was repeated four times with similar results.

MC38-CEA+ cells at multiple E:T cell ratios (Fig. 4C). In a follow-up experiment, anti-FasL was tested at multiple concentrations using a fixed E:T cell ratio (Fig. 4D). At 20 μg/ml, anti-FasL completely inhibited CTL-mediated lysis of irradiated MC38-CEA+ cells, and the extent of inhibition began to titrate out in a dose-dependent fashion. In contrast to what was observed with anti-FasL, the isotype control Ab had no inhibitory effect on tumor cell lysis (Fig. 4D).

Irradiation increases tumor cell sensitivity to lysis by a second, Ag-specific CTL line

To extend the observation that irradiation sensitizes tumor cells to Ag-specific CTL-mediated killing, we tested another CD8+ CTL line with specificity for a wild-type p53 determinant previously found to be functionally expressed by MC38 cells, but not EL-4 cells (40). Since MC38 CEA+ cells were derived from MC38 cells, we assayed them here for sensitivity to p53-specific CTL.

Following irradiation, we observed an increase in Fas expression as well as a significant increase (3-fold; LU = 7.4 for unirradiated vs 20 LU for irradiated at the 12.5:1 E:T cell ratio) in lysis of MC38-CEA+ cells (Fig. 5A). The p53-specific CTL lysed EL-4 cells at a much lower level before or after irradiation (Fig. 5B) despite a dramatic increase in Fas expression postirradiation (6 to 83%). The cause of the increased lysis observed against EL-4 cells at the highest E:T cell ratio is unclear, but may reflect radiation-induced altered expression in p53 as well as other possible phenotypic and molecular events.

Irradiation-induced up-regulation of Fas is maintained on dividing cells for >96 h

As Fas is up-regulated after irradiation (Figs. 2–5), we sought to determine the duration of Fas up-regulation by monitoring expression over several days. To that end, the expression of Fas on both MC38 and MC38 CEA+ cells was monitored up to 96 h postirradiation (Fig. 6, A and B). Interestingly, the expression of Fas increased steadily in both cell populations, showing the highest expression levels (83%) by 96 h. As irradiation can cause effects that may last several generations, we monitored the expression of Fas on the irradiated population of MC38-CEA+ cells as well as on cells arising from cell divisions via two-color Fas/CFSE cell staining (Fig. 6, C and D). The cell populations that were positive for Fas expression (Fig. 6C) showed a steady decrease in CFSE intensity (Fig. 6D), indicating that the dividing cells each expressed increased levels of Fas after irradiation. In this experiment Fas expression was maintained for >96 h and through at least four cell divisions.

Fas is up-regulated on tumor cells in vivo after irradiation

Next we examined whether Fas expression on MC38-CEA+ cells can be up-regulated by localized, external beam irradiation in vivo. To that end, B6 mice were injected with MC38-CEA+ tumor cells s.c. After 9 days, the tumor was irradiated (8 Gy) in one subset of mice, while another subset of mice was maintained without radiation. The tumors were excised 72 h after irradiation and prepared for immunohistochemical staining using an anti-Fas mAb (Fig. 7, A and B). The irradiated tumor (Fig. 7B) demonstrated intense Fas immunoreactivity compared with the nonirradiated tumor (Fig. 7A).
7A). An isotype control Ab (Fig. 7, C and D) was included to demonstrate specificity. Histopathologic analysis revealed that there was no significant contribution of Fas-expressing leukocytes as shown by H&E staining (Fig. 7, E and F; Dr. A. Molinolo, National Institute of Dental and Craniofacial Research, personal communication). In addition, tumors were immunostained with the CEA mAb (COL-1), and no differences were observed between irradiated and nonirradiated cells (data not shown).

Irradiation of tumor cells in vivo enhances the efficacy of CTL adoptive immunotherapy

To examine whether localized, external beam irradiation of s.c. growing tumors improves tumor rejection by CEA-specific CTL in vivo, we made use of an adoptive transfer paradigm. MC38-CEA⁺ cells were injected s.c. on the right hind leg of B6 mice. Nine days later, groups of mice were then divided into those that received no treatment, irradiation of the tumor alone, CTL alone, or the combination of both irradiation of the tumor and CTL. Tumors of mice that did not receive any treatment (Fig. 8A) grew progressively, ultimately causing the death of the animals (100% by day 30). Exposure of the tumors to irradiation on day 9 (Fig. 8B), while appearing to initially cause tumor regression, ultimately failed to significantly impact the extent of tumor growth in these mice (p = 0.069 compared with no treatment). Treatment of tumors with adoptively transferred Flu-specific CTL on day 10 (Fig. 8C) did not significantly inhibit tumor growth (p = 0.3 compared with no treatment). Similarly, treatment of tumors with adoptively transferred CEA-specific CTL on day 10 (Fig. 8D) did not significantly inhibit tumor growth (p = 0.0508 compared with no treatment). Treatment of tumors with the combination of irradiation and CTL adoptive transfer (Fig. 8E), however, resulted in a marked and significant decrease in tumor growth rate and tumor volume (p < 0.0001 vs no treatment; p = 0.0025 vs irradiation alone; p = 0.0048 vs adoptive transfer alone). In addition, 50% of the mice treated with the combination of irradiation and CTL adoptive transfer resolved their tumor mass and remained tumor free for the duration of the experiment (40 days). To validate the concept of the combination of radiation therapy and CTL adoptive transfer, the experiments described above were repeated in a fully homologous system in which the recipient was a CEA-Tg mouse. These mice contain the human CEA transgene under control of the endogenous human CEA promoter and express CEA in normal gastrointestinal tissue and in fetal tissue in a manner similar to that expressed in humans. Again in this study only the combination of irradiation of the transplanted tumor followed by adoptive transfer of CEA-specific CTL resulted in a significant reduction of tumor burden (p = 0.01 vs untreated mice; p = 0.009 vs adoptive transfer alone).

Discussion

In this study we have investigated the phenotypic, functional, and biologic consequences of irradiation of mouse colon carcinoma cells on Ag-specific CTL responses both in vitro and in vivo. Although the conventional rationale for the use of ionizing radiation in neoplastic disease has been to exploit its cytotoxic properties, radiation has also been shown to induce multiple effects on cells and tissues as a function of dosage. Curative (high dose) radiation, however, has sometimes been associated with significant collateral damage of normal tissue. In animal models and more recently in clinical studies, lower doses of radiation may induce immunomodulatory activities by up-regulating MHC molecules, tumor-associated Ag (46, 47), adhesion molecules (14, 48), and death receptors, including Fas (16–18, 49).
Fas and its cognate ligand, FasL, are transmembrane glycoproteins belonging to the TNF receptor and ligand superfamilies, respectively (17). Engagement of Fas by FasL triggers the recruitment of adaptor proteins, followed by activation of the caspase signaling pathway, culminating in physiological cell death. Functional Fas is highly expressed on a variety of nonmalignant tissues, while Fas loss of function has been reported to accompany a malignant phenotype. Multiple molecular mechanisms have been described to account for loss of Fas function in cancer, including down-regulation of transmembrane Fas by promoter methylation, transcriptional repression, histone acetylation, or alternative mRNA splicing to produce soluble Fas protein lacking a transmembrane anchor (50). Fas is a frequent target for inactivation during oncogenesis, and it has been hypothesized Fas-induced apoptosis plays a crucial role in the biology and response of malignant disease (9, 50). We sought to examine whether a sublethal dose of gamma irradiation impacts functional Fas expression in otherwise Fas-resistant or low Fas-expressing tumor cells in vitro and in vivo, and whether this may improve Ag-specific CTL killing via Fas-dependent mechanisms. We also examined the antitumor effects of localized irradiation of tumors in vivo, alone and in combination with these CEA-specific CTL given by adoptive transfer. This latter point also served as proof-of-concept as to whether both external beam irradiation and immune cells can work together to achieve a more intensifi ed antitumor response.

The model system involved a murine adenocarcinoma cell line, MC38-CEA⁺, that expressed low levels of Fas (Fig. 2) and was weakly sensitive to Ag-specific CTL (Fig. 1). In general, perforin-mediated cytotoxicity and Fas/FasL interactions have been characterized as the major effector mechanisms for CTL function (51). The CEA526 CTL line used here can lyse CEA peptide-pulsed targets through perforin mechanisms, as reported by Schmitz et al. (33). However, CTL killing of MC38-CEA⁺-expressing endogenous Ag was not achieved significantly through perforin (33); CTL killing of MC38-CEA⁺ tumor cells was completely blocked by the addition of anti-FasL Ab (Fig. 4, C and D), possibly reflecting the relatively lower strength of signal-1 (i.e., MHC/Ag recognition). This is consistent with the observation of Kessler et al. (52), who demonstrated that TCR signaling that was too weak to elicit perforin-dependent cytotoxicity or cytokine production could induce Fas-dependent cytotoxicity, possibly by translocation of preformed Fas ligand to the cell surface.

In this study it was shown that irradiation up-regulated cell surface expression of Fas and ICAM-1 molecules in both MC38 and MC38 CEA⁺ cells in a dose-dependent manner, but had no enhancing effect on the tumor Ag (CEA) or MHC class I expression, as determined by flow cytometry (Fig. 2). The up-regulation of CEA in clinical tumor samples following irradiation has been reported previously (53). However, the MC38-CEA⁺ tumor cells express CEA under the control of a retroviral promoter (CMV),

**FIGURE 7.** Fas is up-regulated on tumor cells in vivo after irradiation. B6 mice were injected with $3 \times 10^5$ MC38-CEA⁺ tumor cells s.c. After 9 days tumors in a subset of mice were subjected to external beam irradiation (8 Gy) in situ. Tumors were harvested 72 h after irradiation, fixed, sectioned at 5 μm, and immunostained with anti-Fas mAb (A and B) or an isotype control Ab (C and D). Also shown are the corresponding H&E-stained sections (E and F). Photomicrographs are shown at ×40.
FIGURE 8. Irradiation of tumor cells in vivo enhances the efficacy of CTL adoptive immunotherapy. B6 mice were injected with $3 \times 10^5$ MC38-CEA$^+$ tumor cells s.c. A, Mice received no treatment. B, Tumors in mice were subjected to external beam irradiation (8 Gy) in situ on day 2 (Δ). C, Tumors in mice were subjected to external beam irradiation (8 Gy) in situ on day 9 (Δ). D, Mice were adoptively transferred i.v. with $8 \times 10^6$ CEA-specific CTL on day 10 (▲). E, Tumors in mice were subjected to external beam irradiation (8 Gy) in situ on day 9 (Δ), followed by adoptive transfer of CEA-specific CTL on day 10 (▲). Tumor volume was monitored. These experiments were repeated four times with similar results.

which is not responsive to irradiation-induced modulation (39). Irradiation of MC38 and MC38-CEA$^+$ tumor cells up-regulated Fas and ICAM-1 mRNA expression, which was paralleled by increases in cell surface protein expression of those molecules, implying that these radiation-induced effects acted at the level of transcription rather than translation (or translocation). In addition, the up-regulation of Fas was durable; MC38-CEA$^+$ tumor cells exposed to irradiation expressed heightened levels of Fas for $>96$ h (Fig. 6). Interestingly, the dividing cells continued to express the detectable levels of Fas for at least four generations. Furthermore, Fas was biologically active, based on functional Fas assays (Fig. 3) and inhibition of radiation-induced sensitization of MC38-CEA$^+$ targets to CTL-mediated lysis by mAb blocking experiments (Fig. 4). The up-regulation of ICAM-1 following irradiation (Fig. 2) appeared to have no functional role associated with the increased sensitivity of MC38-CEA$^+$ tumor cells to CTL lysis (Fig. 4). This could be due to the low expression levels of ICAM-1 following irradiation (Fig. 2) and does not preclude the possible role of ICAM-1 following irradiation in vivo.

Overall, the ability of radiation exposure to improve the lytic sensitivity of targets to Ag-specific CTL was tested and verified using two distinct CTL lines, one with specificity for a CEA epitope and the other with specificity for a wild-type p53 epitope, suggesting that this outcome was not unique to either effector cell population. In an effort to translate these in vitro findings to an in vivo setting, we conducted adoptive transfer experiments in the CEA model. B6 mice were transplanted s.c. with MC38-CEA$^+$ tumor cells, and after 9 days the tumors were subjected to a single dose of low level external beam radiation. The dose of radiation used was determined to be noncurative; the MC38-CEA$^+$ tumors exhibited a marginal, but insignificant, reduction in growth compared with nonirradiated tumors (Fig. 8). Similarly, the adoptive transfer of CEA-specific CTL did not affect the growth rate of the transplanted tumor. However, the adoptive transfer of these CTL 24 h following irradiation of the tumor showed a significant reduction of tumor volume. In addition, 50% of the mice receiving the combination therapy completely eradicated the tumor mass. The combination of effects of external beam radiation and adoptive T cell transfer was found to be synergistic. These data are consistent with those reported by Ganss et al. (54), in which whole-body lethal irradiation, followed by bone-marrow reconstitution and cycles of adoptive T cell transfer, resulted in tumor destruction of the insulinoma. Tumor destruction was associated with significant inflammation, lymphocytic influx, and re-establishment of normal vasculature within the tumor. Although future studies are warranted to elucidate in detail the nature of the rejection mechanisms described here, we found that Fas was up-regulated on these irradiated tumor cells compared with the unirradiated controls, as determined by immunohistochemistry (Fig. 7). Experiments using transplanted tumor cells defective for functional Fas expression will further determine the role of this molecule in tumor regression.

In addition to radiation, many chemotherapeutic agents used as the standard of care have also been shown to enhance Fas expression (10). These observations thus support the idea that strategies, such as targeted radiation or chemotherapy (10, 55), aimed at inducing death receptor(s) expression on tumor cells may be used to improve antitumor responses via adoptive immunotherapy or active specific immunotherapy. Indeed, adoptive transfer of autologous oligoclonal or clonal T cell populations has been used clinically for antitumor therapy with variable results (56–58). Active specific immunotherapy of cancer via vaccines, in contrast, relies on the in vivo generation and expansion of tumor-specific T cells. Recombinant poxvirus-based vaccines have been developed, such
as recombinant vaccinia virus containing the transgenes for CEA and three T cell costimulatory molecules (B7-1, ICAM-1, and LFA-3, designated rV-CEA/TRICOM) and a replication-defective avipox virus (fowlpox) containing the same four transgenes (designated rF-CEA/TRICOM). It has been demonstrated in preclinical (38, 59) and now clinical (60) 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 endogenous manipulation of relevant cell surface molecules on tumor cells, such as Fas, by localized gamma irradiation may have implications for the combination of immunotherapy and radiation therapy.

Acknowledgments

We thank Diane Poole and Marion Taylor for their excellent technical assistance, and Dr. Alfredo Molinolo (National Institute of Dental and Craniofacial Research) for evaluation of immunohistochemical staining. We thank Debra Weingarten for her editorial assistance with the preparation of this manuscript.

References

TCR signaling, can activate CTL for Fas- but not perforin-dependent cytotoxicity or cytokine production. *J. Immunol.* 161:6939.


